UNIVERSITY OF ALBERTA

Disinfection of biofilm bacteria on drinking water piping surfaces using ozone and monochloramine

by

Li Chang

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

In

Environmental Engineering

Department of Civil and Environmental Engineering

Edmonton, Alberta

Fall 2007

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ISBN: 978-0-494-33213-9
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ISBN: 978-0-494-33213-9

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ABSTRACT

This thesis examines a protocol using ozone and monochloramine for disinfection of biofilm bacteria on cement mortar lined pipe used in drinking water distribution systems. An experimental study was carried out in which biofilms were cultivated on cement surfaces in a laboratory biofilm reactor. The biofilms were exposed to ozone, monochloramine and to sequential combinations of ozone followed by monochloramine. An improved procedure for removal and enumeration of biofilm bacteria from the surfaces was developed and tested. Inactivation of biofilm bacteria by ozone was a non-linear function of the ozone *Ct* product and was limited to 1.7 log at an ozone *Ct* of 120 mg x min/L. In contrast, exposure to a monochloramine *Ct* product of 3,600 mg x min /L resulted in 2.7 log inactivation. Evidence of a synergistic effect when ozone and monochloramine were applied sequentially was inconclusive. The results provide a more rational basis for ozone disinfection of water pipelines in practice and suggested that monochloramine might be an effective disinfectant for biofilm in pipe.

DEDICATION

I dedicate this thesis, and all the effort that went into it, to my husband Hao, for his love, support, inspiration and encouragement, and to my son, Tony, who always showed his cute, lovely and great smile through to the finish of this work.

ACKNOWLEDGMENTS

The completion of this thesis would not have been possible without the contributions and support of many individuals and collectives. First and foremost of these people is my supervisor, Dr. Stephen A. Craik, who provided professional guidance, support, suggestions, and positive criticism through my research project. His attitude of study and science impressed me, and encouraged me to finish the thesis.

I would like to express my sincere appreciation to Dr. Tong Yu, Associate Professor for his full cooperation to work in his laboratory and his intelligence and knowledge in helping my experiment and providing valuable suggestions.

A lot of thanks I would like give to Mrs. Maria Demeter and Mr. Garry Solonynko for their laboratory assistance and guidance. Thanks to Nick Chernuka for helping me to solve computer and printer problems.

Thanks for the great company of Dong Li, Renzhuo Chen, Yangang Feng, Richard Lu, and Faisal Special thanks to Ding Ning for helping hand in carrying out some measurement.

Funding assistance for this project was provided by Denver Water, USA. Special thanks to Mr. Steve Lohman at Denver Water for supporting the project.

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1 Introduction

1.1 Water quality problems in drinking water distribution systems

The proper treatment and protection of clean drinking water is essential for human health. As far back as 1849, Dr. John Snow published information that cholera-causing disease events were spread by sewage-contaminated drinking water in England (Smith, 2002). From then on, waterborne diseases related to contaminated drinking water have been reported frequently from other areas of Europe, North America, Australia and Asia. For instance, typhoid fever is a waterborne disease with several thousand cases per year reported in the early 20th century, however by the 1980's fewer than 300 cases per year were reported (Hrudey and Hrudey, 2004). A cholera epidemic began in Scotland in October 1848 and spread to England in February 1849. It caused more than 6,000 deaths in Scotland and more than 53,000 deaths in England and Wales (Morris, 1976). People have been concerned with the proper disinfection of water for about 150 years (Hrudey and Hrudey, 2004). Chlorine is one of the most widely used disinfectants because of its effectiveness, its ease of use, and its low cost. Through several decades of research and practice by scientists and engineers, the conventional water treatment method of filtration combined with chlorination has almost eliminated waterborne outbreaks in major North American cities during the early years of 20th century (Hrudey et al. 2004). However, waterborne disease outbreaks continue to occur even in the developed world. In North America, more than 30 waterborne disease outbreaks were reported over the period from 1970 to 2000 (Hrudey and Hrudey, 2004). The Milwaukee, Wisconsin, water crisis,

caused by *Cryptosporidium parvum*, happened in 1993 and an estimated 4,400 people were affected and possibly 50 to 70 were died (Hoxie *et al.*, 1997).

Inadequate repair or installation of water distribution systems has caused or has been associated with a number of water borne disease outbreaks (Herwaldts *et al.*, 1991). Kirkmeyer *et al.* (2002) reviewed that more than 35 cases of waterborne disease outbreaks associated with contamination of water pipes that were reported from 1920 to 1990. He indicated that disease outbreaks might result from cross contamination during breaks or repairs of water mains followed by insufficient disinfection prior to returning the main piping to service. During the repair and installation processes, pathogens and indicator microorganisms such as fecal coliform that are present in soil and trench water at construction sites can enter into pipes. Such bacteria may become established within a distribution system by re-establishment, perhaps as a biofilm (Charles *et al.*, 2000). Therefore, disinfection of mains post repair or installation is an important public health protection measure.

1.2 Current water main disinfection standard methods and their limitations

The purpose of potable water disinfection is to prevent transmission of waterborne disease through the inactivation of pathogenic microorganisms. Generally, the disinfection process in water distribution systems was considered satisfactory if coliform bacteria were not detected. The current industry standard for disinfection of water mains involves the application of highly concentrated free chlorine for several hours, according to the "AWWA Standard C-651 for Disinfection Water Mains" (American Water Works Association, 2005). Three methods are included and introduced

in this standard: the tablet method, the continuous-feed method, and the slug method. The choice of methods generally depends upon a given situation. The length and diameter of the main, the type of joints present, the availability of materials, the equipment required for disinfection, the training of personnel who will carry out the disinfection procedure and safety influence the choice of methods. The tablet method is suitable for mains with diameters of 24 inches (1200 mm) or smaller. During installation or repair, hypochlorite granules or tablets are attached on the inside wall of the main. Subsequently, the main is filled with potable water when installation is completed. According to the C-651 standard, an initial chlorine concentration of 25 mg/L and a minimum contact time of 24 hr are required for adequate disinfection. The required free chlorine residual of 25 mg/L must be detectable after 24 hr of exposure. This method is only used when the main is kept dry and clean during construction. The continuous-feed method consists of preliminary flushing to remove particles and eliminate air pockets. Then main is filled with potable water and is exposed to a flowing chlorine solution with a concentration of at least 25 mg/L free chlorine. When the entire main is filled with heavily chlorinated water, chlorine application can be stopped. The water is retained for 24 hr in the entire pipe to ensure adequate disinfection. The free chlorine residual should not less than 10 mg/L after the disinfection period. This method is appropriate for large diameter mains. For even larger pipes, and to meet the needs of situations requiring reduced contact times this procedure can be modified using free chlorine concentrations up to 100 mg/L for shorter periods. This is called the slug method. The flow of water is stopped when the residual concentration of chlorine drops below 50 mg/L. The slug method is preferred because this method requires only 3 hr of contact time or even shorter when a higher

concentration of chorine is used. After final flushing, a water sample should be collected and tested for fecal coliform bacteria or heterotrophic bacteria. Although the contact time and chlorine concentration of each these methods are not identical, they should all result in the elimination of coliforms. If the initial disinfection fails, that is, if the coliform test is positive, the new main must be flushed and re-sampled. If the check sample also fails, the mains need to be rechlorinated by the continuous-feed or slug method until no *fecal coliforms* are detected or Heterotrophic Plate Count (HPC) bacteria are less than 500 colony forming units (CFU)/mL. The pipe wash water should be dechlorinated using a chemical such as sodium bisulfite and then discharged.

Though chlorine-based disinfection methods for water distribution system repair and installation are conventionally used and are generally effective in eliminating coliforms, they have several limitations (Schulz *et al.*, 2004) that are listed below.

- The water main disinfection methods described above lack a rational scientific basis such as the concept of concentration - contact time product (Ct) that is used for predicting disinfection performance in drinking water treatment plants (WTPs). The operation procedure relies on experience only. Adequate disinfection depends on the fecal coliform detected after final flushing. So far, there have not been any studies to show that 25 mg/L chloramines for 24 hr exposure in repaired or installed water mains can ensure undetectable fecal coliforms after disinfection of drinking water piping.
- Due to the lack of a rational scientific basis, the chlorine dose rates may be overly conservative. The heavily chlorinated water from the disinfection of the main must be dechlorinated prior to discharge in a body of water. In a recent survey of

industry practice, this heavily chlorinated water was not dechlorinated before disposal or release to the environment in 87% of cases (Haas et al., 1998).

- The on-site preparation and transportation of hazardous chemicals such as chlorine gas, granular hypochlorite and granular or liquid sodium bisulfite (sodium bisulfite is used to remove (neutralize) any residual chlorine after disinfection) is a time-consuming procedure that is also a potential source of environmental damage.
- For the tablet method, there is no way to ensure a uniform distribution of chlorine residuals from the dissolving tablets into the entire water volume within the main. Sometimes, the tablets are not fully dissolved and this may possibly result in under-dosing or inadequate chlorine contact for portions of the pipe.
- Both chlorination and dechlorination steps require two separate trips to the pipeline disinfection site, each taking about 4 hours to complete. Moreover, a 24 hr contact time is required to ensure adequate disinfection. These time-consuming procedures can be problematic for construction contractors with tight schedule constraints.
- USEPA and CCME (Canadian Council of Ministers of the Environment) are considering decreasing the threshold standard of the chlorinated discharges to the environment.

As an alternative disinfectant, ozone has been used to inactivate suspended bacteria and other microorganisms in potable water systems for over 100 years (Donlan, 2000). Several laboratory studies have confirmed that ozone is an effective disinfectant

against encysted protozoa, such as cysts of *Giardia lamblia* and oocysts of *Cryptosporidium parvum* (Gyürék *et al.*, 1999, U.S. Environmental Protection Agency, 1999, Facile *et al.*, 2000, Li *et al.*, 2001b). Also, ozone does not produce the same chemical by-products as chlorination (Singer, 1990). Although use of ozone for drinking water disinfection has grown rapidly in North America and Europe in recent decades, very limited research has been conducted on the use of ozone for disinfection of water mains following installation or repair. Further research should be conducted to develop better disinfection methods for the disinfection of water mains. In particular, further research should be conducted to determine if ozone is more suitable than chlorine for water main piping disinfection and to establish a rational basis, like the *Ct* concept used in water treatment plant disinfection processes, for use of ozone as a pipeline disinfectant.

1.3 Hypotheses and Objectives

Denver Water, USA is conducting a major research project and field trial to evaluate the use of ozone for disinfecting water mains. They have been using a trailer-mounted flow-through ozone system and a hypochlorination system for water main disinfection since 2003. This project is divided into three overlapping phases (Schulz *et al.*, 2003):

- Phase I (Laboratory study). The purpose of this phase is to determine ozone demand, to compare the effect of disinfection using ozone and chlorine, to establish *Ct* tables for biofilm HPC bacteria inactivation and to develop a computer model to predict the required ozone dose to meet *Ct* requirement under different water quality conditions.

- Phase II (Pipe loop study). The purpose of this phase is to determine ozone and chloramine demand and decay characteristics in an operating pipe system, to evaluate the impact of new and old (turberculated) pipes on disinfection performance, to evaluate the performance of a prototype ozone generation/injection system and to develop standard operating procedures for ozone disinfection of water mains.
- Phase III (Full-scale verification study). The purpose of phase III is to design and construct a permanent, easy-to-operate and feasible truck-mounted ozone generation/injection system and refine the standard operating procedure under Phase II.

The research described in this thesis is a part of the Denver Water laboratory study. An initial laboratory study was carried out by Mathrani (2006) as part of his M. Sc. thesis research, however, several limitations were identified in his work. The objective of this thesis research was to develop solutions to the limitations of Mathrani's work and to build upon his initial research. Specifically, the hypotheses of the thesis research were:

- Physical and chemical methods might help to remove and elute the bacteria from the surface of the coupon, thus providing a more accurate enumeration of biofilm bacteria colonizing the cement surface.
- A relationship exists between biofilm HPC bacteria disinfection and the average concentration-time product ($C_{avg}.t$) in aqueous solution to which the biofilm is exposed. This relationship may or may not be linear. If such a

relationship can be determined and quantified, it would be useful for developing field procedures for disinfecting cement-mortar line pipe.

- Sequential treatment using ozone followed by monochloramine might result in a synergistic effect on inactivation of biofilm HPC bacteria. If the synergistic effect can be measured and quantified, this could be further used to optimize field procedures for disinfecting cement-mortar lined pipe.

The objectives of the thesis research were:

- To improve and optimize biofilm cultivation conditions on cement-lined mortar (CML)-coated coupons in an annular biofilm reactor;
- To develop a protocol for detaching biofilm HPC bacteria from the CML coupons and to enumerate the detached bacteria;
- To investigate the ozone demand and provide a rational basis for disinfectant *Ct* values and microorganism reduction in terms of inactivation of HPC bacteria in the biofilm;
- To develop a kinetic model to predict ozone biofilm inactivation efficiency as a function of ozone *Ct* values
- To investigate relatively low monochloramine concentrations (i.e. 2 mg/L) and sequential disinfection using ozone followed by monochloramine for inactivation HPC bacteria in the biofilm

2 Literature Review

2.1 Biofilms in the aquatic environment

2.1.1 Biofilm terminology and structure

In their 1990 book, Characklis and Marshal described the structure of biofilms: "Microbial cells attach firmly to almost any surface submerged in an aquatic environment. The immobilized cells grow, reproduce, and produce extracellular polymers which frequently extend from the cell forming a tangled matrix of fibers which provide structure to the assemblage termed a biofilm"(Characklis and Marshall, 1990). During the last decade a new conceptual model of heterogeneous biofilms has been presented. According to the new model, biofilms are made of microcolonies separated by interstitial voids. Lewandownski (2000) described that heterogeneous biofilm were composed of 1) densely compact sublayers, 2) roundly-shaped microcolonies, 3) streamers, and 4) interstitial voids.

Figure 2-1 illustrates a model heterogeneous biofilm. Microcolonies are compact aggregates of extracellular polymers containing densely packed microorganisms. The sublayer consists of dense and discontinuous microcolonies that are attached to the substratum. They are separated by interstitial voids and form a network of interconnected channels. Water can freely move through the network structure of interconnected channels of biofilm and convey nutrients and other materials required for cell growth. The top sublayer consists of roundly-shaped microcolonies similar in appearance to a mushroom. The porosity of the bottom layer is much lower than the porosity of the upper layer when the biofilm is immature. As the biofilm gets older, the semi-continuous

sublayer tends to grow denser and thicker. Streamers are like antenna and may function to disseminate and elongate the microcolonies.

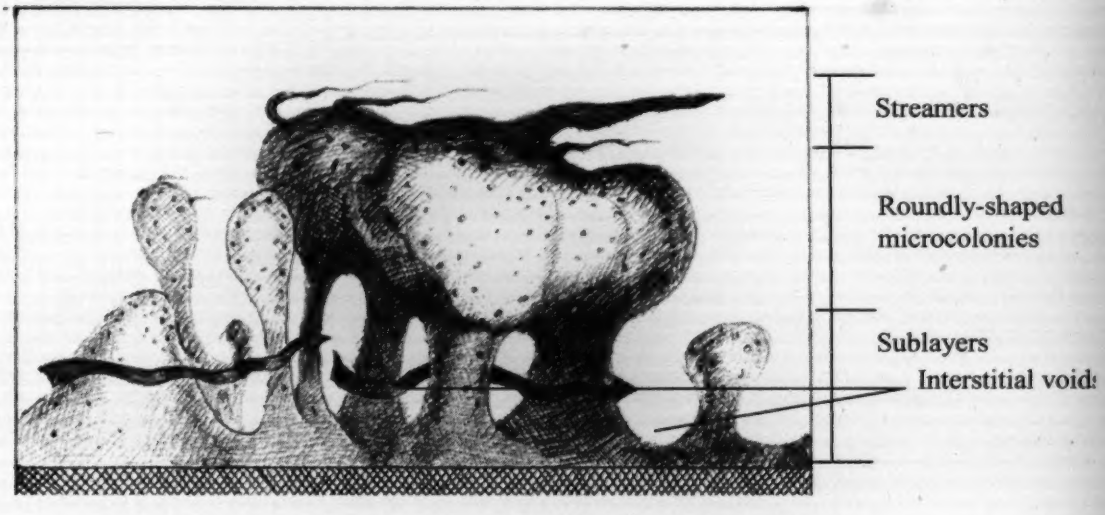

Figure 2-1 The biofilm system structure (Adapted from Lewandowski, 2000)

2.1.2 Biofilm development and characteristics

Bacterial biofilms are composed of surface-attached cells and extracellular polymeric substances (EPS) excreted from the cells. The biofilm structure provides an optimal environment for cooperative behavior among cells, such as exchange of genetic material or efficient substrate utilization (Donlan, 2002). The EPS matrix provides biofilm mechanical stability by filling and forming the space between the bacterial cells and helping them coalesce (Korstgens *et al.*, 2001).

Biofilm development proceeds in several stages wherein cells initially adhere, divide and colonize on a surface (Danese *et al.*, 2001). In their review paper, Lazarova

and Manem (1995) suggested that initial cell adherence and biofilm formation plays an important role and has a considerable impact on the structure and physicochemical properties of the "mature" biofilm. A number of physical, chemical and biological phenomena influence bacterial biofilm formation. Hörsch *et al.* (2005) indicated that certain y-proteobacteria have attachment mechanisms which help form the primary biofilm. Other bacteria may subsequently colonize. The y-proteobacteria surround themselves with an exopolymeric film and recruit more cells to form microcolonies interspersed within fluid-filled channels. A number of genes for cell envelope, substrate transport and metabolism as well as various regulatory factors have been implicated in biofilm formation (O'Toole *et al.*, 2000, Di Martino *et al.*, 2002, Heydorn *et al.*, 2002, Wen and Burne, 2002). Once developed, it is difficult to remove biofilms from the substrate completely (Simoes *et al.*, 2003).

2.1.3 Mechanisms of resistance of biofilm to disinfectants

The research regarding the biofilm resistance to reduction by chemical disinfectants focuses mainly on two aspects: the biofilm exterior environment and the biofilm matrix itself. Attachment of bacteria to surfaces is considered one of the most important factors that contributes to increased resistance of bacteria to chemical disinfectants in the exterior environment (LeChevallier *et al.*, 1987). For instance, surface-attached, unencapsulated *Klebsiella pneumoniae* grown on glass microscope slides are 150-fold more resistant to disinfectants like hypochlorite and monochloramine compared with suspended bacteria. Other factors that affect resistance to disinfectants are biofilm-bulk connection surface, age of the biofilm, encapsulation of the bacteria,

composition of the substrate material, environmental conditions (pH, temperature, hydraulic status, turbidity), nutrient availability etc.. All of these factors contribute to the resistance of bacteria to free chlorine. deBeer *et al.* (1994) further indicated that exposure to very concentrated chlorine solutions inhibits the respiratory pathway of biofilm bacteria. Thus the limited penetration of chlorine into a biofilm matrix is likely to be another important factor influencing the biofilm reduction efficacy. The interface between biofilm and bulk fluid seems to be the only factor affecting resistance to monochloramine as deBeer et al. (1994) reported. Other research reveals that the resistance provided by one mechanism could be multiplied by a second mechanism (Stoodley *et al.*, 1994, Heinzel, 1998).

Cloete (2003) systematically reviewed disinfection resistance mechanisms from the aspect of the biofilm matrix as follows:

1. *Limited diffusion of antimicrobial agents through the biofilm matrix*; Charged (ionic) interactions between the glycocalyx and the antimicrobial agent, an increase in the distance the agent must diffuse, molecular sieving (size exclusion), and the viscosity of the glycocalyx can contribute to limited diffusion of disinfectants through a biofilm.
2. *Interaction of the antimicrobial agents with the biofilm matrix (cells and polymer)*; A disinfectant must first bind to the bacterial cell surface and then must pass through the cell wall (gram positive) or outer membrane (gram negative) to reach the site of bactericidal action at the cytoplasmic membrane or within the cell cytoplasm. In gram positive bacteria there are no specific receptor molecules or permeases to assist or block disinfectant

penetration, therefore intrinsic resistance of gram positive bacteria to disinfectants is low. In contrast, the gram negative cell envelope has evolved to prevent the passage of substances in and out of the cell with a remarkable degree of specific receptor molecules.

3. *Enzyme mediated resistance*; Enzymes within bacterial cells (intracellular enzymes) can transform disinfectants into a non-toxic form due to their biodegradation function. A host of phenolic, aromatic and other compounds that are toxic to many bacteria can be degraded by certain bacterial enzymes.
4. *Level of metabolic activity within the biofilm;* It has also been suggested that the increased resistance of biofilm bacteria to disinfectants occurs because some of the cells within a biofilm experience a nutrient limitation that causes them to enter a slow-growing or starved state (Brown *et al.*, 1988). Under these conditions, there can be considerable variation in the composition of the bacterial cell envelope, ultimately affecting the barrier properties. During exposure to concentrated chlorines, cells near the biofilm-bulk liquid interface lose or cease their respiratory activity, while respiratory activity continues to persist deep in the biofilm-bulk liquid interface. Slow or non-growing cells are more resistant to a variety of antimicrobial agents when compared with cells grown in rich media at high specific growth rates.
5. *Genetic adaptation*; in the long term, at least some of the cells in a biofilm adopt a distinct and relatively protected biofilm phenotype. This genetic

adaptation results in an increased resistance of the biofilm bacteria to disinfectants.

6. *Efflux pumps;* Cells of the biofilm have intricate efflux pump systems that can function to rid cells of a wide range of seemingly dissimilar compounds. The gene *aqc* A and *aqc* B in the cell of *Staphylococcus aureus* is a QAC (Quarternary Ammonium Compound) efflux system. In this example, cells of the biofilm appear resistant to QAC.
7. *Outer membrane structure*; The mechanism of adaptation of the cell envelope provides resistance to antimicrobial agents. For disinfectants to be effective, they must be able to penetrate the cell envelope and attain a sufficiently high concentration at the target. Hydrophilic antibacterial agents (such as hypochlorous acid, HOCl, the active ingredient in chlorinated water) are prevented from entering through the outer membrane of the cell by the presence of hydrophobic functional groups on the outer membrane proteins.

2.2 Heterotrophic Plate Count Bacteria: Methods and Monitoring in Drinking Water

Heterotrophic microorganisms, which are broadly defined as microorganisms that require organic carbon for growth, comprise an important group of microorganisms found in a water supply. Heterotrophic microorganisms include bacteria, yeasts and moulds. Some researchers have found that increases in the numbers of heterotrophic bacteria in the distribution system after the water leaves the treatment plant (Geldreich *et al.*, 1985)

and in the water treatment devices mounted at the household tap (Reasoner *et al.*, 1987) are often the result of bacterial re-growth following disinfection. Some authors have expressed concerns regarding the public health risk of some of these heterotrophic bacteria (McFeters *et al.*, 1986, Payment *et al.*, 1988). It has been suggested that individuals in high-risk categories, such as immuno-compromised individuals, could be at risk from the consumption of drinking water due to the presence of opportunistic pathogens that are members of the heterotrophic group (Rusin *et al.*, 1997).

To count the total number of heterotrophic bacteria present in water, a variety of simple culture-based plate-count methods designed to recover a wide range of microorganisms are utilized. These tests are collectively referred to as "heterotrophic plate count" or "HPC" (Bartram *et al.*, 2003b) and were formally referred to as the "total plate count". Since Robert Koch published a historic paper that introduced the application of microbial indicators for surveillance of water hygiene in 1883 and described the methodology for HPC measurement in water (Bartram *et al.*, 2003b), it has been wildly accepted that HPC enumeration is helpful in assessing changes in finished water quality during water main system repair and installation of new distribution system piping (LeChevallier *et al.*, 1990, Butterfield *et al.*, 2002, Bartram *et al.*, 2004, Stine *et al.*, 2005).

Although HPC measurement has evolved over more than a hundred years and has been formalized by standard methods for the examination of water and wastewater (Eaton *et al.*, 1995), the procedure still involves a wide variety of test conditions that lead to a range of quantitative and qualitative results. For example, the employed test incubation temperatures range from around 20°C to 40°C, incubation times range from a few hours

to seven days or a few weeks, and nutrient levels range from low to high. The test itself does not specify the organisms that are enumerated; all microorganisms that produce a visible colony on the test agar (i.e. colony-forming units - CFU) are counted. Only a small proportion of the metabolically active microorganisms present in a water sample may grow and be detected under any given set of HPC test conditions, and the population recovered will differ significantly according to the method used (Bartram *et al.*, 2003a). The US Environmental Protection Agency (U.S. EPA) has suggested that HPC bacteria in drinking water should not exceed 500 colony-forming units per millilitre (CFU/mL), primarily because of the concern over interference with *coliform* detection (U.S. Environmental Protection Agency, 1989). However, the high HPC by themselves are not considered to be indicative of fecal contamination or to necessarily or represent a public health risk. This is very different from the total or fecal coliform indicators. Fecal coliform are sometimes detected at very low levels in distribution system piping after repairs. While they are a useful field indicator of fecal contamination and public health risk, these bacteria are difficult to use in experimental disinfection studies because they are not likely to be present in sufficient numbers to allow disinfection efficiency to be measured to high levels (i.e greater than 1 or 2 log reduction). HPC bacteria are typically much more plentiful. This is the reason that HPC bacteria were chosen for this experimental study. The underlying assumption is that biofilm coliform bacteria, or more importantly pathogens in the biofilm, will be inactivated to the same level or greater than biofilm HPC bacteria when exposed to ozone and monochloroamine.

In this study, the HPC bacteria were enumerated at 20°C for 7 days on R2A agar using a membrane filter method. The results of Geldreich (1990) indicated that R2A agar yields significantly higher bacterial counts than Plant Count Agar (PCA). McFeters (1990) reported that incubation times longer than the standard method specified time of 48 h (i.e from three to seven days) lead to higher viable HPC at 20°C to 37°C in drinking water. Reasoner and Geldreich (1985) found that the temperature of incubation has a significant effect on the HPC results of any plate counting method. For drinking water samples, incubation at a temperature lower than 30°C for a long time period generally yields higher bacterial counts than incubation at 37°C for shorter periods. The HPC data of Reasoner and Geldreich (1985) illustrates the effect of incubation temperature on HPC results. They found that the measured HPC bacteria concentration determined in drinking water samples using any medium increased as the incubation temperature decreased from 35°C to 28°C to 20°C. The plate count method is limited to sample volumes of 10 mL. In contrast, the membrane filter method permits analysis of samples from ‹ 1.0 mL to 10.0 L (Eaton *et al.*, 1995). The membrane filter method, therefore, provides and advantage over the pour plate method in terms of sensitivity to low HPC bacteria concentration.

2.3 *Ct* values are an important indicator for microorganism reduction.

Disinfection efficiency or microorganism reduction is often expressed in terms of the inactivation, *I*, which is defined in log units by the following equation:

$$I = -\log\left(\frac{N}{N_0}\right)$$ Equation 2-1

where:

N = number of viable organisms after exposure to a disinfectant

N_0 = number of viable organisms before exposure to disinfectant

Drinking water can be disinfected of bacteria and pathogenic microorganisms by contact with chemicals such as chlorine, chloramines, ozone, etc. A relationship between disinfection and contact time was originally described by Chick in 1908 (Haas, 1999). Her research determined that as contact time between water and disinfectant increases, the survival ratio of (N/N_0) decreases. Watson modified Chick's equation to include the effect of disinfectant concentration in addition to time. The resulting Chick – Watson Law is given by:

$$I = -\log(N/N_0) = kC^n t$$ Equation 2-2

where:

C = concentration of disinfectant (mg/L)

n = coefficient of media attributes (e.g., dilution, pH)

t = contact time (minutes)

k = rate constant $((L/mg)^n min)$

Based on this equation, microorganism survival is seen to be a simple function of disinfectant concentration and contact time. If the constant n is assumed to be unity,

survival is then proportional to the *Ct* product. This *Ct* concept is broadly used as a design and operational parameter for disinfection of suspended microorganisms in water and wastewater treatment. The term has also been accepted to evaluate bacteria inactivation in many other fields such as water distribution systems, the food industry and in dental health.

By comparison of Equations 2-1 and 2-2, it is clear that inactivation is a function of the *Ct* product. If the disinfectant concentration is not constant, an average concentration, is often used (C_{avg}) and inactivation is considered to be function of the $C_{avg}t$ product.

If n is 1, then log inactivation is a linear function of the *Ct* (for $C_{avg}t$) product. In many situations, observed log inactivation is not a simple non-linear function of the *Ct* product and *C* and *t* have separate effects. The Hom model (Gyürék and Finch, 1998) is a modification of the Chick-Watson model that includes exponents on both *C* and *t*. Hom equation expresses the relationship between inactivation and *Ct* as shown below:

$$I = -\log(N/N_0) = kC^n t^m \qquad \text{Equation 2-3}$$

2.4 Biofilm control technology

Food industry

Biofilms have been of considerable interest in the context of food hygiene, medicine and dentistry. Various preventive and control strategies can be applied in an effort to control biofilm formation on food contact surfaces. These include the design of food plant hygiene procedures, design of equipment, choice of materials, and selection of detergents and disinfectants coupled with physical methods (Kumar and Anand, 1998).

Bacteriocins (a type of bactericide) and enzymes are gaining importance and have a unique potential for effective biocontrol and removal of biofilms in the food industry. These newer biocontrol strategies are considered important for the maintenance of bio-film-free systems and for quality and safety of foods (Kumar and Anand, 1998). Ozone (O_3) is a strong antimicrobial agent with numerous potential applications in the food industry. High reactivity, penetrability, and spontaneous decomposition to a nontoxic product (i.e., O_2) make ozone a viable disinfectant for ensuring the microbiological safety of food products. Ozone has been used successfully to inactivate contaminant microflora on meat, poultry, eggs, fish, fruits, vegetables, and dry foods (Kim *et al.*, 1999). The gas is also useful in detoxification and elimination of mycotoxins and pesticide residues from some agricultural products (Kim *et al.*, 1999).

Yuk *et al.* (2006) conducted experiments to determine the effects of ozonated water (at 1, 3, and 5 mg/L aqueous ozone concentration) alone at different exposure times (0.5, 1, 3, or 5 min), or in combinations of 3 mg/L ozone with 1% organic acids (acetic, citric, or lactic acids) during 1-min exposure for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* on lettuce. Results showed that 5 mg/L ozone treatment for 5 min resulted in 1.09 log and 0.94 log inactivation of *E. coli* O157:H7 and *L. monocytogenes*, respectively, indicating insignificant reductions. In comparison, treatment with 3 mg/L ozone combined with 1% citric acid for 1 min resulted in 2.31 and 1.84 log reductions, respectively. Citric acid is a naturally occurring and popularly used preservative that prevents the growth of bacteria, fungi and other microorganisms. In addition, it is an environmentally benign cleaning agent that can act as an antioxidant for the food industry. In addition, after the combined ozone-citric acid treatment described

above followed by storage at 15°C for 10 d and subsequent packaging, the bacterial populations increased by approximately 9.0-log CFU/g. This indicates that ozonated treatment does not have a residual antimicrobial effect during storage. Park *et al.*(2005) examined the effects of ozone exposure on the growth of indigenous microorganisms in Enoki mushrooms using the same disinfectant, concentration and contact times that Yuk et al. (2006) used. Populations of mesophilic bacteria, yeasts and molds in Enoki mushrooms appeared to decrease after stepwise increases in ozone concentration (1 to 5 mg/L) or exposure time (0.5 to 5 min). There were reductions of 1.03 to 2.61 log CFU/g in mesophilic bacteria and of 1.21 to 2.75 log CFU/g in yeasts and molds in all ozone-treated enoki mushrooms. Combination of 3 mg/L ozone and 1% citric acid brought about significant reductions in both mesophilic bacteria (3.52 log CFU/g) and fungi, yeasts and molds (2.77 log CFU/g) on the surfaces of enoki mushrooms. These experiments confirmed that the disinfection ability of ozone plus citric acid is much stronger than either ozone or citric acid alone for disinfection of food. That is, ozone and citric acid result in a synergistic disinfection effect.

Oztekin *et al.* (2006) studied inactivation of microbial flora on dried figs. Ozone gas was applied for 3 or 5 hours at 5 or 10 mg/L. Results indicated that a minimum treatment of 5 mg/L for 3 hours was required to reduce the microorganism count on the dried figs in their study condition. Decreases in total aerobic mesophyllic microorganisms, yeast/mould counts and *coliform* bacteria were approximately 38%, 72% and 100%, respectively, at this ozone treatment level.

Oral microorganisms

Bacterial biofilms have been recognized in dental plaque (Marsh and Bradshaw, 1995). Few articles reported effective methods on how to eliminate biofilm microorganisms growing on the surface of teeth. Nagayoshi *et al.*(2004) reported that ozonated water strongly inhibited the accumulation of experimental dental plaque *in vitro*. They found that almost no viable bacterial cells were detected after dental plaque samples from human subjects were exposed to ozonated water (4 mg/L) for 10 seconds. Walker *et al.*(2003) studied many disinfectants to reduce biofilm bacteria attached to the inner surface of tubing of a dental unit water system. They found that ozone did not completely eliminate the viable bacteria from the surfaces (65% reduction in biofilm total viable count). Alternatively, they found that commercial hydroxide-containing biocidal products such as Sporklenz, Sterilex Ultra, Dialox, Sterilox, Sanosil, Oxigenal, and Grotanat Bohterbad resulted in a 100% biofilm TVC reduction and more than 95% reduction in biofilm coverage. These chemicals are extremely powerful cleaning, disinfectant and descaling products that may be used for all haemodialysis machines, water treatment and other units. They are not appropriate for drinking water treatment.

2.5 Biofilm control in drinking water distribution systems

2.5.1 Application of chlorine and chloramines for biofilm control in distribution systems

2.5.1.1 Disinfection chemistry and mechanisms of chlorine action in drinking water

Gaseous chlorine or solutions of calcium or sodium hypochlorite are generally used for water disinfection. They can be rapidly hydrolyzed in aqueous solution to form the strong oxidants hypochlorous acid (HOCl) and the hypochlorite ion (OCl^-). HOCl and OCl^- are called free available chlorine. The following equations show the reactions of three compounds in pure water:

$$\text{Gaseous chlorine: } Cl_2\,(g) + H_2O \rightarrow HOCl + H^+ + Cl^-$$

$$\text{Calcium hypochlorite: } Ca(OCl)_{2(s)} + 2H_2O \rightarrow Ca^{2+} + 2HOCl + 2OH^-$$

$$\text{Sodium hypochlorite: } NaOCl\,(aq) + H_2O \rightarrow Na^+ + OCl^- + H_2O$$

Depending on the pH value, HOCl can dissociate to produce the OCl^- ion:

$$HOCl \leftrightarrow H^+ + OCl^-$$

These free available chlorine species can both inactivate bacterial cells and can react with extracellular polymeric substrates (EPS) in biofilms (Armon *et al.*, 1997). McFeters and Camper (1983) studied how these compounds affect bacteria. They found that free chlorine reacted with cell surface enzymes and reduced the metabolic activities of bacteria, resulting in a reduction in an inability to transport glucose and amino acids through the cell membrane. Dychdala (2001) suggested that bacteria are possibly inactivated due to a reaction between available chlorine species and amino groups present

in the cell membrane or in the cytoplasm. This results in cell death. HOCl and OCl^- have very distinctive disinfectant behaviors. OCl^- is repelled by negatively charged bacterial cell walls. Therefore, HOCl is generally a stronger disinfectant than OCl^-.

2.5.1.2 Disinfection chemistry and mechanisms of action of chloramines in drinking water

Monochloramine is a weaker oxidant, and is normally considered to be a less effective disinfectant than free chlorine. In dilute aqueous solution (1 to 50 mg/L), chlorine reacts with ammonia in a series of bimolecular reactions to form monochloramine:

$$NaOCl + H_2O \rightarrow Na^+ + OCl^- + H_2O$$

$$H^+ + OCl^- \leftrightarrow HOCl$$

$$HOCl + NH_3 \rightarrow NH_2Cl + H_2O$$

$$HOCl + NH_2Cl \rightarrow NHCl_2 + H_2O$$

$$HOCl + NHCl_2 \rightarrow NCl_3 + H_2O$$

These competing reactions are dependent upon pH and the relative chlorine to nitrogen (Cl_2: N) concentration. The reaction between HOCl and NH_3 will convert all free chlorine to monochloramine at pH 7 to 8 when the Cl_2 : N weight ratio is 5:1 or less (LeChevallier and Au, 2004).

Ingols (1958) examined the reaction of monochloramine with several amino acids and tripeptides and concluded that monochloramine inactivates bacteria cells by reacting primarily with membrane-bound enzymes.

2.5.1.3 The application and effect of chorine and chloramine on biofilm reduction in drinking water distribution systems

LeChevallier *et al.* (1987) reported that maintaining a 1.0 mg/L free chlorine residual was insufficient to control *coliform* occurrences in distribution system biofilms. The statistical model they developed demonstrated that biofilm HPC levels can be controlled to less than 100 CFU/mL (given pH 8.0 and temperature of 23°C) in drinking water utility in New Jersey when a chlorine residual of 1.2 mg/L is kept for one hour. LeChevallier *et al.* (1988) compared the disinfection efficiencies of chlorine and chloramines against suspended and biofilm HPC bacteria. Biofilm HPC bacteria were cultivated on the surfaces of granular activated carbon particles, metal coupons (copper, lead, and wild steel), and glass microscope slides placed in an experimental distribution system – a 1 L wide-mouth jar with low nutrient water at 1 to 2°C. The biofilm HPC bacteria was composed of 70% *Pseudomonas picketti*, 18% *Moraxella* and 12% *Pseudomonas paucimobilis*. They found that the disinfection resistance of biofilm HPC bacteria against free chlorine was 150 to 3000 times greater than that of HPC bacteria suspended in the bulk water. In contrast, the disinfection resistance of biofilm HPC bacteria against monochloramine was only 2 to 100 times more than that of suspended HPC bacteria. They concluded that monochloramine penetrates and kills biofilm HPC bacteria more effectively compared to free chlorine. LeChevallier *et al.* (1990)

investigated disinfection of biofilm growing on the inside walls of iron pipes filled with treated drinking water in an experimental pipe system. The results of this study indicated that attached HPC bacteria did not show any significant change in viability after exposure to water containing 4 mg/L free chlorine for two weeks. In contrast, suspended bacteria were inactivated by more than 3 log after the same treatment. Biofilm growing on other pipe materials tested such as copper, galvanized and PVC were readily inactivated by a 1 mg/L residual of free chlorine or monochloramine. LeChevallier *et al.* (1990) also reported that a synergistic effect was observed when two disinfectant chemicals were applied sequentially to biofilms grown on iron pipe, although in most cases there was no change in the inactivation of the biofilm. In some experiments, they observed nearly a 1 log inactivation increase when the pipe was exposed to 4 mg/L free chlorine for two weeks followed by 4 mg/L monochloramine for an additional two weeks. Even greater inactivation (about 4.5 log total inactivation) was achieved when the pipe was exposed to monochloramine first, followed by exposure to free chlorine. However, they were not able to repeat the results in subsequent experiments.

A study conducted by Clark *et al.* (1994) reported that the concentration of biofilm bacteria attached to pipe surfaces increased when the disinfectant residual disappeared following ozone, chlorine or monochloramine treatment. They also reported that there was greater than 2 log inactivation of biofilm HPC bacteria exposed to 2.5 mg/L concentrations of chlorine, or chloramine, or ozone, and a concentration of 19.43 mg/L of hydrogen peroxide at different contact times. In this study, monochloramine and hydrogen peroxide resulted in greater inactivation of HPC bacteria than the other disinfectants because of the long-lasting residual concentration provided by these

disinfectants. Monochloramine and hydrogen peroxide are less reactive than ozone and free chlorine, and therefore they are better able to penetrate biofilms and inactivate bacteria deep within the biofilm. Momba *et al.* (1998), however, reported that residuals of hydrogen peroxide (16.5 mg/L), monochloramine (1 mg/L) and free chlorine (0.2 mg/L) were not enough to prevent the formation of biofilms on the surfaces of stainless steel and cement coupons which were installed in a laboratory-scale distribution system simulation unit.

Gagnon *et al.* (2006) compared disinfection efficacy of chlorine at a *Ct* of 30 mg x min/L to chloramines at a *Ct* of 60 mg x min/L for inactivating biofilm HPC bacteria grown on polycarbonate coupons. Chlorine outperformed chloramines for controlling suspended HPC bacteria in the bulk water (2.1 to 2.7 log inactivation for chlorine vs. 1.3 to 1.4 log inactivation for chloramines), while both disinfectants resulted in similar inactivation of bacteria grown on cast-iron (CI) coupons (1.2 to 1.3 log removal). Both disinfectants resulted in comparable biofilm inactivation on polycarbonate (1.5 to 1.9 log removal) and cast-iron coupons (1.1 to 1.2 log removal). Gagnon *et al.* (2006) also examined the impact on bacterial growth after switching from free chlorine or monochloramine to chlorine dioxide in a model drinking water distribution system test reactor with a chlorine dioxide *Ct* of 15 mg x min/ L. Switching from free chlorine to chlorine dioxide did not significantly change suspended HPC bacteria levels in the bulk water or biofilm HPC bacteria for either polycarbonate or cast-iron coupon pipes. After switching from chlorine to chlorine dioxide, suspended HPC bacteria concentration was reduced by 0.2 log in both cast-iron and polycarbonate systems, and biofilm HPC bacteria concentration was reduced by 0.1 to 0.3 log. However, chlorine dioxide led to

approximately a 10-fold reduction in suspended bacteria levels in the chloraminated reactor, while biofilm HPC counts were not significantly affected (0.2 to 0.3 log removal).

Momba and Makala (2004) compared biofilm formation on various plastic-based pipe materials (polyvinyl chloride-PVC, unplasticized polyvinyl chloride-UPVC, medium density polyethylene-MDPE) and cement-based pipe materials (cement and asbestos cement)) in laboratory-scale systems. The pipe materials were installed in a reactor containing treated surface water (water treatment plant) before being exposed to 2.5 mg/L chlorine for 20 minutes followed by 1.5 mg/L monochloramine for 24 h. Bacterial regrowth occurred on the surface of all pipe materials between 168 and 672 h. The capability of bacterial regrowth occurring on the surface of test pipe materials was linked to the depletion of the concentration of monochloramine residual. Statistical evidence showed that the type of pipe materials greatly influenced the density of biofilm bacteria. Cement-based materials- supported significantly less fixed bacteria than plastic-based materials. Momba and Makala (2004) also found that physical-chemical parameters including temperature, pH, turbidity, dissolved organic carbon, total nitrogen, and sulphate did not have a significant impact on biofilm bacteria concentrations. This implies that the presence of a monochloramine residual in the bulk water is one of the most important factors in controlling biofilm formation on different pipe materials.

2.5.2 Application of ozone for biofilm control in water distribution system piping

2.5.2.1 Ozone characteristics and reaction mechanism in aqueous solution

The application of ozone for disinfection of drinking water was introduced over a century ago. The use of ozone as a primary disinfectant was not very popular in North

America, however there has been renewed interest recently because of the emergence of chlorine-resistant pathogens such as *Giardia* and *Cryptosporidium*. Ozone is usually generated by the corona discharge technique. In this process, dry and pure oxygen gas is passed between two electrically charged, narrowly-spaced electrodes. At a specific voltage and current, a portion of the oxygen is converted to ozone (Carlins and Clark, 1982). Ozone is an unstable gas and can react with substances directly and indirectly in aqueous solution. As such, ozone can oxidize compounds either as molecular ozone or by hydroxyl free radicals produced during the decomposition of ozone. These different reaction pathways lead to different oxidation products and are controlled by different reactions kinetics (Gottschalk *et al.*, 2000). Some of the chemical properties displayed by ozone may be described by the aqueous auto-decomposition reaction showed below:

$$O_3 + H_2O \rightarrow HO_3^{\bullet} + OH^-$$

$$HO_3^{\bullet} + OH^- \rightarrow 2HO_2^{\bullet}$$

$$O_3 + HO_2^{\bullet} \rightarrow HO^{\bullet} + 2O_2$$

$$HO^{\bullet} + HO_2^{\bullet} \rightarrow H_2O + O_2$$

The free radicals, $HO_2^{\bullet}$ and $HO^{\bullet}$, have great oxidizing powers and are probably the active form in the disinfection process (Tchobanoglous *et al.*, 2003). Besides the oxidizing power, another major reason to choose ozone as a disinfectant is that ozone does not form chlorinated disinfection by-products. For example, chlorination of drinking water results in the formation of chlorinated by-products such as trihalomethanes (THMs) that have been associated with various detrimental human health effects (Gopal *et al.*, 2007). Ozone does not produce chlorinated by-products, however it does produce a range of

oxidation by-products such as aldehydes and ketones; these have uncertain effects on human health (Glaze, 1987).

The exact mechanisms of inactivation of bacteria by ozone are not fully understood due to a lack of experimental information, however the following mechanism has been proposed: Ozone reacts first with cell membrane biomolecules, such as glycoproteins, glycolipids and certain amino acids (eg. tryptophan). Also, ozone can disrupt enzymatic activity by reacting with certain extracellular enzymes. Finally, molecular ozone passes through the cell wall and cell membrane, and reacts with the nuclear material (U.S. Environmental Protection Agency, 1999).

2.5.2.2 Disinfection of Suspended Microorganisms Using Ozone

Ozone is considered very effective for killing suspended bacteria. Ozone has also been shown to be effective against chlorine-resistant microorganisms such as *Giardia lamblia* and *Cryptosporidium parvum* and viruses. Korich *et al.* (1990) reported that *Cryptosporidium parvum* is inactivated when the oocysts protozoan were exposed to ozone, chlorine dioxide, chlorine, or monochloramine. The results showed that ozone and chlorine dioxide more effectively inactivated oocysts than chlorine and monochloramine. Greater than 90% inactivation was achieved by treating oocysts with 1 mg/L of ozone for 5 min. Exposure to 1.3 mg/L of chlorine dioxide yielded 90% inactivation after 1 h, while 80 mg/L of chlorine and 80 mg/L of monochloramine required approximately 90 min for 90% inactivation. Researchers suggested that with a possible exception of ozone, the use of disinfectants alone should not be expected to inactivate *C. parvum* oocysts in drinking water. However, ozone inactivation kinetics also revealed a strong temperature

dependency. Li *et al.*(2001a) reported that the *Ct* required to achieve a *C. parvum* inactivation efficiency of approximately 99 percent with ozone at 1°C was approximately 40 mg x min/L, or approximately 12 times greater than that required at 20°C.

The inactivation kinetics of *C. parvum* oocysts with free chlorine and monochloramine after ozone pretreatment have been shown to be significantly faster compared to the kinetics with free chlorine and monochloramine. Driedger *et al.* (2001) investigated the synergy of sequential disinfection with ozone followed by monochloramine at temperatures in the range of 1 to 20°C. They reported that the *Ct* required for 2 log *C. parvum* inactivation with monochloramine at 1°C could be reduced from approximately 64,600 mg x min/L, when using this disinfectant alone, to 1350 mg x min/L, when using monochloramine after ozone pre-treatment at a *Ct* of 22.5 mg x min/L. The conclusion was consistent with results from Rennecker et al. (2000) when they conducted inactivation experiments using ozone, followed by monochloramine or chlorine to inactivate *C. parvum*. The synergistic effect occurred at lower temperatures. Based on the research results generated by a number of studies on ozone inactivation of *C. parvum* in recent years, it is now included in the US EPA Long Term 2 Surface Water Treatment Rule (LT2SWTR) (EPA, 1989, 2003) as an acceptable technology for inactivating *C. parvum* in drinking water.

Ozone also was used to inactivate HPC suspended bacteria in drinking water. In a bench-scale study, Gyürék and Finch (1998) reported 2 to 4 log inactivation of HPC bacteria suspended in 0.05 M pH 6.9 phosphate buffer at 22°C when ozone doses ranged from 0.31 to 2.13 mg/L with contact time of 0.58 to 14.93 minutes.

2.5.2.3 Disinfection of biofilm bacteria using ozone.

Although there are many studies on the effect of ozone for disinfecting microorganisms suspended in water, there are only few studies on the effect of ozone for inactivating microorganisms attached to surfaces. In one set of studies on the use of ozone for disinfection in industrial cooling water systems, Viera *et al.* (1993) showed that exposure to an aqueous ozone concentration of 0.5 mg/L for 10 minutes resulted in 2 log inactivation of biofilm *Pseudomonas fluorescens* bacteria attached to stainless steel and carbon steel surfaces. Viera *et al.* (1999) later assessed the efficacy of dissolved ozone against pure *P. fluorescens* bacteria biofilms grown on carbon steel and stainless steel coupons in quiescent conditions. An aqueous ozone concentration of 0.35 mg/ L with 30 to 60 minutes contact time resulted in 3 log reduction of biofilm *P. fluorescens*. They also found that ozone not only inactivated biofilm bacteria, but also caused detachment of sessile bacteria from the metal surface. In another study, the same group of researchers (Viera *et al.*, 1996) reported that a change from quiescent to turbulent flow was associated with a 1 log increase in biofilm bacteria inactivation by aqueous ozone.

Very limited research has been conducted on ozone utilization to inactivate attached bacteria in drinking water distribution system piping. Mathrani (2006) studied inactivation of HPC biofilm grown on cement mortar line CML coupons in a biofilm reactor. The source of the bacteria was water used to wash pipes stored outside in the Denver Water pipe yard. After the biofilm developed, the coupons were removed from the bioreactor for disinfection using ozone or free chlorine. Mathrani (2006) reported that inactivation of suspended HPC bacteria was 3 to 4 log for an aqueous ozone *Ct* ranging from 6.2 to 62.6 mg x min/L at pH 6.0 and 6.9 and at 22°C. However, inactivation of

attached bacteria in thick, relatively mature biofilms was limited to 1 log at the same *Ct*, pH and temperature conditions. Attached HPC bacteria in thinner, less mature biofilms, treated under the same conditions, resulted in an apparent negative log inactivation values when the ozone *Ct* was low. Mathrani concluded that a reaction-diffusion limitation might have resulted in ozone concentrations at cell walls or membranes of bacteria within the biofilm that were much lower than in the bulk solution. This mechanism, however, does not explain the negative inactivation that was observed at low *Ct*. Further, according to his experimental design and operation procedure, there were some limitations in his experiment that led to the unexpected results.

- Mathrani carried out disinfection experiments using CML coupons that were removed from the bioreactor with no pre-treatment and, sometimes, the biofilms were with relatively thick and well-developed. In actual field operations, during main break repairs or replacement of pipes, the loosely attached, surface layers of biofilms are usually removed from the inside pipe surfaces using high pressure water and are then flushed from the system. Therefore, Mathrani's disinfection experiments on fully developed biofilms may not have been representative of actual pipeline disinfection operations.
- The experimental ozone disinfection reactor used by Mathrani was designed as a semi-batch reactor. When the reactor flask was filled to capacity with aqueous ozone solution, the disinfection experiment had to be terminated. Using this procedure, it was difficult to develop and maintain constant ozone residual in the reactor and the aqueous ozone *Ct* values that could be achieved was limited.

- Mathrani did rigorously investigate the procedures used to remove and disperse the bacteria from the CML coupon surfaces. Enumeration of the HPC bacteria, therefore, might not have been accurate.

2.6 Summary of literature review

Free chlorine, chloramine and ozone have classically been used as disinfectants to inactivate bacteria in drinking water. Most of the research reviewed above focuses on inactivation of suspended bacteria in drinking water. Ozone, chlorine and chloramine show differing efficacies with ozone being the most powerful and chloramine being the least. In particular, ozone is very effective at inactivating *Cryptosporidium* and *Giardia*, while chlorine and monochloramine are less effective. In recent decades, attached bacteria in the distribution system have been considered a risk for public health. However, few studies have been conducted in this field. Although a disinfection method for repaired and new installed water mains is recommended in "AWWA Standard C-651 for Disinfection Water Mains", the main disinfection methods lack a rational scientific or design such as the (*Ct*) concept that is used for water treatment process design. The operation procedure only relies on the operational experience. It is pertinent to investigate the true relationship between *Ct* and biofilm bacteria inactivation. Based on previous studies and inactivation mechanisms, ozone and monochloramine are potentially effective against biofilm bacteria, however, little information is known regarding the ozone *Ct* products required to achieved biofilm bacteria reductions. A previous study on inactivation of biofilm bacteria grown on cement mortar was limited by experimental procedures and was inconclusive. For monochloramine, the presence of a

monochloramine residual in the bulk water is one of the most important factors in controlling biofilm formation. The results of some research also display that the synergistic effect can be observed using ozone followed by monochloramine for inactivation of suspended *C. parvum* in drinking water at low temperature. Sequential disinfection for biofilm bacteria in distribution system is rarely reported. This knowledge gap should attract more research attention.

3 Experimental Methods and Materials

3.1 Summary

Mathrani (2006) conducted laboratory experiments in which he used chlorine and ozone to inactivate suspended HPC bacteria and biofilm HPC bacteria that were grown on cement surfaces. The sources of bacteria were water samples used to wash pipes in the Denver Water pipe yard. However, his ozone disinfection experiments produced some unexpected results, such as negative inactivation of biofilm bacteria that are difficult to explain. As a follow-up to Mathrani's work, the research contact herein addresses the previous experimental problems. The specific experimental objectives of this research are to:

a. develop a better method to remove biofilms quantitatively from cement coupons and disperse them evenly into buffer solutions. This would improve the accuracy of disinfection experiments;

b. use the improved method to determine inactivation of the biofilm bacteria by aqueous ozone and to establish the relationship between biofilm inactivation and aqueous ozone concentration-time product (*Ct*) values;

c. evaluate inactivation of the biofilm bacteria using aqueous monochloramine at concentrations typically found in a drinking water distribution system (ie. 1 to 2 mg/L as total chlorine) and to compare this to ozone inactivation;

d. evaluate inactivation of biofilm bacteria by a sequential treatment using ozone followed by monochloramine and to determine if there is synergistic effect of the two disinfection methods on biofilm inactivation.

As with the experiments of Mathrani et al. (2006), the HPC bacteria concentration measurement were used as the principal measure of live bacterial concentration in the biofilm.

This research included two experiments. Experiment I included biofilm cultivation on cement coupons in an annular biofilm reactor, and testing of methods to remove the biofilm and disperse HPC bacteria into a solution for enumeration. Experiment II consisted of the biofilm disinfection experiments with ozone, monochloramine and ozone followed by monochloramine. The wash water samples were mixed with phosphate pH 6.2 buffered de-ionized water. The buffer pH of 6.2 was chosen such that it was equal to the pH of pipe wash water samples provided by Denver Water. The volumetric ratio of the pipe wash water and phosphate buffer was determined by the target organic strength of the water to which the biofilms were to be exposed as measured by chemical oxygen demand (COD). An annular biofilm reactor was connected to the reservoir and the mixed solution flowed through the reactor. Biofilms were allowed to develop on cement coupons by immersing them into the mixed solution in the reactor for specified periods of time. Coupons were removed from the reactor and the loosely attached biofilm was removed from the cement surface. The coupons were then placed in a disinfection reactor and disinfectants, either ozone, monochloramine, or both, were then applied. The efficiency of biofilm disinfection was determined by measuring attached HPC bacteria concentration on the cement surface of coupons that were exposed to disinfectants and comparing this to the HCP bacteria concentration on the cement surface of similar coupons that were not exposed to disinfectant. Detailed experimental procedures are described in the following sections.

3.2 Pipe wash water samples

The sources of biofilm bacteria used in this research were samples of water that were used to wash the interior of cement-mortar line (CML) cast iron drinking water pipes. The pipes were stored in the Denver Water (Denver, CO) pipe yard and were washed prior to installation in the distribution system during distribution main repairs or replacement projects. The chlorine residual in the wash water was quenched using a reducing agent to ensure that there was no chlorine residual remaining. The wash water samples were collected by Denver Water personnel and were shipped directly from the Denver Water Quality Lab to the Environmental Engineering department lab of University of Alberta (UA) by overnight courier. In the Environmental Engineering Lab at UA, the water samples were filtered using a coffee filter to remove most of the larger particles. COD, turbidity, pH, free chlorine of the water samples were measured. The samples were then stored in a 20 L carbuoy at 4°C until they were used in experiments. The samples were aerated periodically by air sparging to avoid development of anaerobic conditions and were used within one week of arrival at the UA lab.

3.3 Biofilm cultivation

3.3.1 Biofilm reactor system

The biofilm reactor system consisted of an annular biofilm reactor (Model 1230LJ Annular reactor, Montana Biosystems, Montana, US.) that could accommodate up to twenty 15 cm ×1 cm rectangular cement coupons. The coupons were actually plastic that were coated with a cement mortar composed of Portland cement and find sand. They

were purchased from Montana Biosystems (the same supplier as the bioreactor). The experimental apparatus for growing biofilm samples on the cement mortar coupons consisted of the biofilm reactor, a reservoir, a peristaltic pump, an air supply system, air filters and connection tubes and fittings (See Figure 3-1). Before inoculation, the biofilm reactor containing 20 coupons was sterilized by filling with 70% non-denatured ethyl alcohol and soaking overnight followed by several rinses with sterile de-ionized water. A pipe wash water sample was mixed with 0.05 M phosphate buffer (pH = 6.2) to make a solution with a COD of approximately 150 mg/L. The mixture was then poured into a 10-L sterilized transparent carbuoy which served as the reservoir. Water was pumped from the bottom of the carbuoy via a peristaltic pump (Model 7553-70, Cole Parmer) through neoprene tubing (inside diameter 7.9 mm) into the top of the biofilm reactor through tygon tubing (inside diameter 3.1 mm). The water flow rate was controlled at 100 mL/min, and was monitored with an in-line flowmeter. The water left the bottom of the reactor and flowed back to the top of the reservoir by gravity. Air was introduced continuously into the reservoir through a sparger submerged below the water surface. The air was provided by the compressed air supply system of the NREF (Natural Resources Engineering Facility) building at UA and was filtered via a HEPA filter (Whatman Inc. Clifton, NJ, U.S.) to remove microorganisms and other particulate contaminants. The reservoir contents were mixed continuously via a stir plate and magnetic stir bar. Addition of the air with mixing helped to ensure dissolved oxygen saturation and aerobic growth conditions within the biofilm reactor. A motor (Model 1120LS, Montana Biosystems) fitted to the top of the biofilm reactor rotated the center part that held the concrete coupons. The rotation rate was 10 rpm and was controlled by

a Regrowth Model 1100 Motor Controller (Montana Biosystems). The rotation ensured a consistent and continuous supply of dissolved oxygen and nutrients to the biofilm cultivating the surface of the cement coated coupons in the reactor. Another tube was connected to the top of the reservoir to act as a vent. This tube was fitted with a HEPA–filter to prevent microbial contamination from laboratory air. This tube was also connected to the reactor effluent tubing to prevent siphoning of water from the reactor.

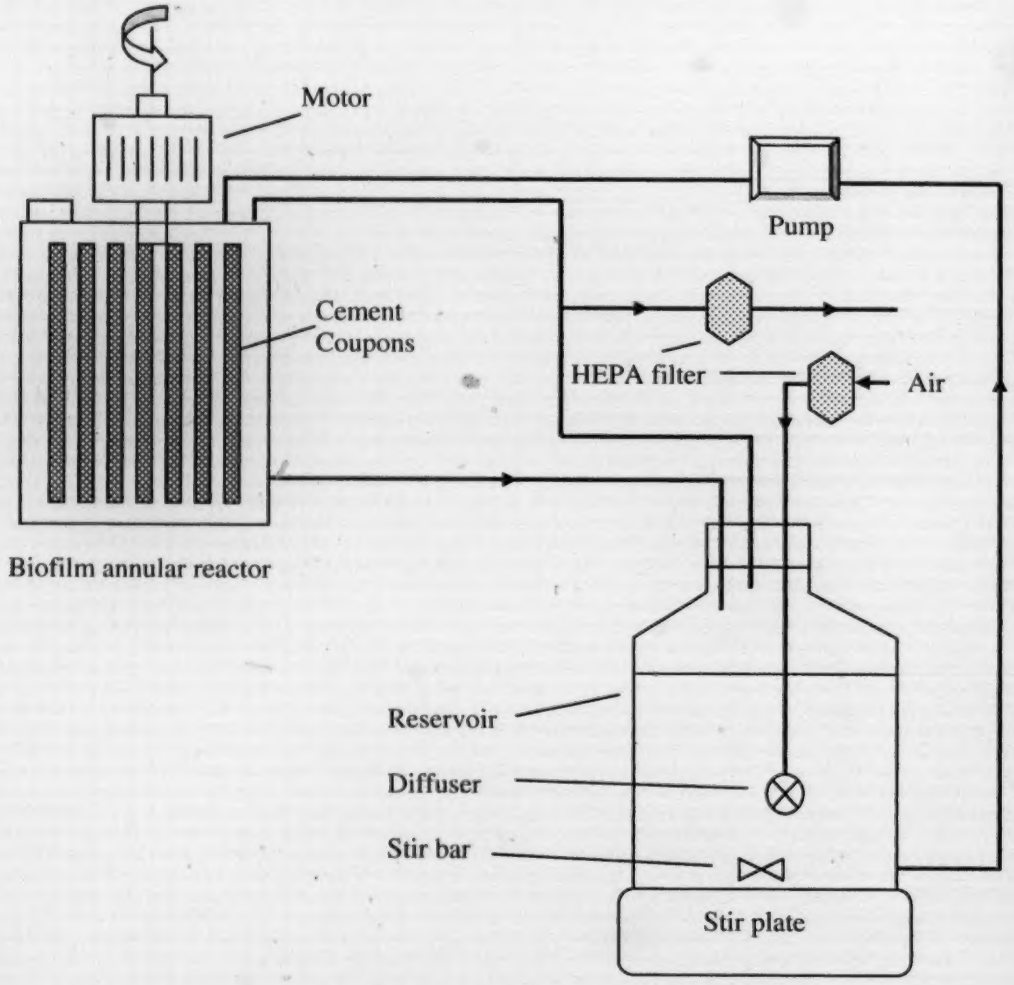

Figure 3-1 Schematic of the biofilm annular reactor operational setup

3.3.2 Parameters controlled and analysis methods

The pH, turbidity, temperature, dissolved oxygen (DO) and COD of the bioreactor recirculation water was monitored regularly by collecting samples from the bottom sample port of the reservoir. The analytical methods are described below:

1) pH

pH was measured using a Fisher Scientific AR 50 pH meter two times a week. The objective of the pH measurement was to ensure that the proper pH range for heterotrophic bacteria growth (from 6.0 to 9.0), was maintained. The target pH 6.2 in the reactor at the beginning of the experiment was based on the pH of pipe wash water samples delivered from Denver Water.

2) Turbidity (NTU)

Turbidity is an expression of the optical property that causes light to be scattered at 90° rather than transmitted through a water sample. It is an indirect measure of the concentration of suspended solids and was used to monitor the suspended bacterial concentration in this study. Turbidity was measured using a Digital Direct-Reading Turbidimeter (Orbeco-Heelige, Farmingdale, NY) and was reported as nephelometric turbidity units (NTU) according to Standard Method 2130 (Eaton, 1995). Turbidity in the bioreactor was measured twice a week.

3) COD (mg O_2 / L)

Chemical oxygen demand (COD) was used as a measure of the dissolved or suspended organic substrate concentration in the bioreactor. The concentration of dissolved organic

substrates determines the rate of biofilm growth. COD therefore, is a critical control parameter in the cultivation of biofilms. COD is defined as the oxygen equivalent of the organic material in a sample that is susceptible to oxidation by a strong chemical oxidant (APHA, 1995). The COD measurement includes not only biodegradable organic substrates, but also non-biodegradable organic substrates as well. Two methods are commonly used to measure COD; the open reflux and the closed reflux method, as described in Standard Method 5220 D (Eaton, 1995). The latter method was employed in this research to measure total (soluble + insoluble) COD in water samples collected from the bioreactor. Basically, a suitable volume of sample and reagent ($K_2Cr_2O_7$, H_2SO_4, $HgSO_4$, Ag_2SO_4 and DI-water, see Standard Methods) was added to a test tube. The tube was inverted several times until homogenous, and put into a reactor for 2 hours to digest at 140°C. Subsequently, the sample was cooled prior to measuring the absorbance of the tube contents at 600nm wavelength. COD was calculated according the measured absorbance and compared to a standard curve developed using known concentration KHP solution in advance of these experiments. The apparatus used for COD measurement was a HACH COD reactor (HACH company, S/N: 981100018386, Loveland, CO) and a spectrophotometer for measuring colour (Pharmacia Biotech Novaspec II at 600 nm). Since the sample was not pre-filtered before analysis, the total (soluble + insoluble) COD was measured.

Two batches of experimental biofilms were cultivated as part of this research. The first batch was used to test methods for removing and enumerating biofilm HPC bacteria from the cement coupons. The second batch was used in the ozone and monochloramine disinfection experiments. For the first biofilm batch, the goal was to maintain the COD

value in the bioreactor recirculation water at approximately 150 mg/L while for the second biofilm batch the goal was to maintain the COD at 50 mg/L. These targets were dictated to some extent by the COD concentration in the pipe wash water samples provided by Denver Water. In each biofilm cultivation experiment, COD was measured once a week in the bioreactor.

4) Dissolved oxygen (mg O_2 / L)

Dissolved oxygen (DO) is an important water quality characteristic. In particular, it was important to ensure aerobic conditions in the bioreactor. It was therefore an important factor to monitor. DO in the recirculation water in the reservoir was measured twice a week using a Fisher Scientific Model 50B dissolved oxygen meter according to the Standard Method 4500–OG (Eaton, 1995).

5) Temperature (^{o}C)

Many parameters, such as DO and pH change when temperature changes. In addition, bacterial growth rates are determined by temperature; therefore temperature control is very important for this experiment. Temperature of the recirculating water in the reservoir was measured twice weekly using a digital thermometer (Tracable, Suzhou China) according to Standard Method 2550 B (Eaton, 1995). The temperature of the bioreactor was maintained at ambient lab temperature of approximately 20°C.

3.4 Biofilm removal, dispersal and enumeration

3.4.1 Biofilm removal

During replacement of piping for renewal or repair of distribution system mains, the interior surfaces of the new pipe sections are often washed or flushed prior to installation. Denver Water uses a high pressure spray nozzle for this step prior to installation, disinfection and flushing. Much of the loose biofilm growth on the interior surfaces of the pipe will likely be removed by the spray wash step before the pipe wall is exposed to disinfectant. The remaining biofilm bacteria will likely be growing mostly in surface cracks, holes and crevices that provide shelter from the effects of the spray. To attempt to match the operation in the field, the loose outer layer of biofilm bacterial growth on the cement coupon surfaces was first removed by gentle scraping with a soft rubber policeman. The scraped coupons were then rinsed by dipping three times in test tubes filled with 50 mL of 0.05 M, pH 6.2 phosphate buffer solution to remove any loose biofilm remaining.

To remove the more firmly attached biofilm for enumeration, one half of each coupon was the submerged in 50 mL of either Camper's dispersion buffer (see section 3.4.3) or pH 6.2, 0.05 M phosphate buffer (see section 3.4.4) in a 140 mL beaker. The coupon surface was brushed using a sterilized medium stiffness toothbrush and any material removed was collected into the buffer. Another 50 mL of the same type of buffer was used to rinse the brushed half of the coupon and the toothbrush. This buffer solution was combined with the first 50 mL solution. The goal of this operation was to transfer as much of the residual biofilm bacteria as possible, from the coupon into the

buffer solution. Thus a 100 mL biofilm/buffer suspension was produced and was enumerated for HPC bacteria (see Section 3.5).

3.4.2 Dispersal of Biofilm HPC Bacteria

After transferring the coupons into the 100 mL buffer solutions, the suspended bacteria were dispersed using a tissue homogenizer (Powergen 700D, Fisher Scientific). The objective was to disaggregate the biofilms and disperse the bacteria, producing a homogenous suspension of bacteria. This process was important to ensure that the number of HPC bacteria in the suspensions was enumerated accurately and that the assumption that 1 colony forming unit arises from one bacterium was accurate. The homogenizer cutting tip was placed into the middle of the 100 mL suspension contained in a 140 mL beaker. The speed of the cutting tip was set at either 13,500 rpm or 25,000 rpm for this study. Coloured dye tracer tests were used to show that the suspension mixed completely throughout the beaker within seconds at these cutting tip settings. Homogenization proceeded for 2, 6, or 10 minutes. The homogenization procedure was done using either Camper's eluting solution as described above or using phosphate buffer with no eluting solution. Thus, a total of 12 different experimental conditions were compared for dispersion efficacy. Each condition was tested twice in order to determine the best set of bacterial dispersal conditions.

3.4.3 Camper dispersion buffer

Camper et al. (1994) investigated a total of 19 different solutions for eluting and enumeration of the HPC bacteria and *E coli* attached to granular activated carbon (GAC). The solution described below as Camper's solution was reported to result in the highest enumerated HPC bacteria for similarly treated samples. The Camper's solution was prepared by dissolving 1.404 g Trizma HCl, 0.134 g Trizma Base (Sigma Chemical CO. Lot: 37H5737, St. Louis, MO.), 0.01% peptone (Fisher Scientific, Lot No: 053238) solution and 10^{-6} M Zwittergent 3-12 (Calbiochem, Lot: 693015, La Jolla, CA.) into de-ionized water from a Milli-Q water system (QTUM000EX, Millpore Quantum™ EX, Sparks, MD) to form a 1000 mL solution. The pH was then adjusted to 7.0 using 1N NaOH or 1N HCl.

3.4.4 Phosphate buffer

The phosphate buffer was prepared by dissolving Na_2HPO_4 (2.36 g/L) and KH_2PO_4 (4.53 g/L) into Milli-Q water to achieve a molar phosphate (PO_4) concentration of 0.05 M. The pH was adjusted to 6.0 or 6.2 if necessary using 1N NaOH or 1N HCl.

3.5 Enumeration of HPC Bacteria

3.5.1 R2A Agar plate preparation

9.1 g of R2A agar powder (Difco™, 218263) was dissolved into 500 mL of Milli-Q water in a 1000 mL Erlenmeyer flask. The flask opening was covered with aluminum foil and was autoclaved at 121°C for 15 mins. About 5 mL of molten agar was

aseptically dispensed into 47 mm diameter pre-sterilized plastic Petri dishes (Fisher Scientific, Whitby, Ontario) in a biosafety cabinet. The dishes were covered and left to cool. The plates were inverted and stored at 4°C for no more than 2 weeks before use.

3.5.2 Preparation of peptone dilution bottles

A 0.1% peptone solution was prepared by dissolving 4 g of peptone powder (Fisher Scientific, Lot No: 053238) into 4 L of Milli-Q water in an Erlenmeyer flask. 90 mL of peptone solution was dispensed into clean milk dilution bottles (Kimax* Brand glass sampling bottle, cat no. 02-945-10, Fisher Scientific) using a Brewer automatic pipetting machine (Division of Baltimore Machine and Equipment Inc. Lot No: 60480, Baltimore, Maryland). The bottles were capped and were autoclaved at 121°C for 20 minutes. In order to avoid injuring HPC bacteria, the bottles were cooled down to room temperature before using.

3.5.3 Preparation of HPC bacterial dilution series

10 mL of the homogenized biofilm suspension was transferred asceptically into a milk dilution bottle filled with 90 mL of a sterile 0.1% peptone solution to create a 10^{-1} dilution. The bottle was shaken well and 10 mL of the 10^{-1} dilution was transferred asceptically to another milk dilution bottle containing 90 mL of sterile peptone to produce a 10^{-2} dilution. This operation was repeated to produce a dilution series. Three dilution series were prepared for each sample. Selected dilutions were chosen for enumeration based on the estimated concentration of HPC bacteria in the homogenized

biofilm suspension. These dilutions were then filtered through black, gridded 0.45 μm pore size 47 mm diameter filters (Fisher Scientific, Lot No: HABG047S1) using a vacuum filtration apparatus. After rinsing with sterile peptone buffer, the filters were individually placed on the R2A agar plates. The plates were covered, inverted and incubated at 20°C for 7 days (See section 2.2). The number of colony forming units on each plate was then counted and the concentration of HPC bacteria in the original suspension was determined by dividing by the dilution. Figure 3-2 shows how the dilutions were prepared.

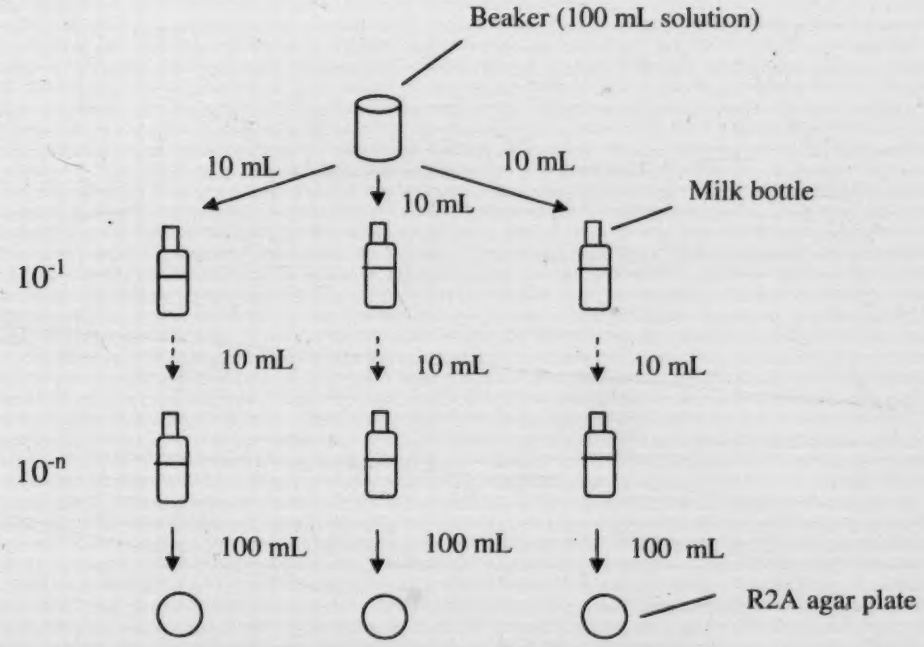

Figure 3-2 Preparation of an HPC dilution series in triplicate

3.5.4 Measurement of biofilm HPC bacteria concentration

Standard method 9215A (Eaton, 1999) suggests that a colony density of 20 to 200 per filter can be accurately counted. In this study, triplicate dilutions were filtered and counted. For each dilution series, three different dilutions were used to prepare plates. If colony numbers were within the range of 20 to 200 in only one dilution, the number of bacteria from the original sample was calculated based on the arithmetic average of the triplicate plates. If colony numbers within the range of 20 to 200 happened in two dilutions, the number of bacteria in the original sample was the arithmetic average of the colonies on the six plates of both dilutions, accounting for dilution factors as needed. The concentration of biofilm HPC bacteria on the coupon surface in colony forming units per cm^2 (CFU/cm^2) was then calculated based on the surface area of the coupon scraped and volume of wash buffer collected (100 mL).

3.6 Biofilm Disinfection Experiments

3.6.1 Experimental Design

Ozone, monochloramine and ozone followed by monochloramine were used as disinfectant treatments in this research. The reason for using the combination of ozone and monochloramine is that monochloramine has been shown to be a more effective disinfectant of biofilms, presumably due to its superior biofilm-penetration ability, than free chlorine (LeChevallier, et al. 1990). It is also the disinfectant that is present in the treated water in the Denver Water distribution system. During pipeline repair work, a pipe that has been disinfected using ozone would be flushed using distribution water

containing a monochloramine residual of approximately 2 mg/L as total chlorine. The hypothesis was that ozone, which is strong oxidant, may react with EPS and weaken the biofilm structurally, thus allowing monochloramine, a more stable chemical, to better penetrate the biofilm. This mechanism would result in a synergistic effect. Ozone disinfection experiments were carried out in Reactor I and Monochloramine disinfection experiments were carried out in Reactor II.

3.6.2 Disinfection with Ozone

3.6.2.1 Ozone generation and collection

The ozone generation and collection system is shown in Figure 3-3. Ozone gas was produced by passing extra-dry oxygen (Praxair, Edmonton, Alberta) through a solid-state, air cooled corona discharge ozone generator (Absolute Systems, Edmonton, Alberta,). The gas pressure at the inlet to the generator was regulated to 10 psi using a pressure regulator. The back-pressure on the generator was controlled at 9.5 psi by means of a needle value on the outlet of the generator and was monitored by a pressure gauge. The resulting gas flow rate was at 0.8 m^3/min and was monitored by a rotameter. The voltage of the generator was set at 65 V. The ozonized gas from the generator flowed through a 3 mm inside diameter Teflon tube into a Pyrex glass wash bottle equipped with a porous glass diffuser (Prixer, USA). The wash bottle was filled with 500 mL of ozone demand-free (ODF) 0.05 M phosphate buffer (pH = 6.0). The wash bottle was placed in an 8.5 L plastic cooler filled with ice-water to cool the water to about 2°C and to minimize the rate of ozone decomposition. The whole system was placed in a fume hood to protect workers against exposure to ozone gas.

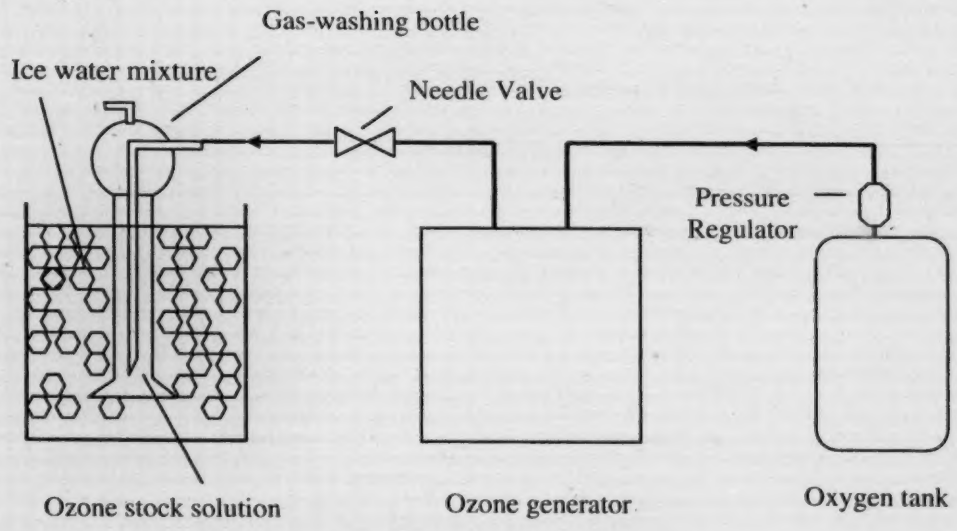

Figure 3-3 Generation of aqueous ozone stock solutions

3.6.2.2 Ozone demand-free water and phosphate buffer

Ozone demand-free (ODF) water was used to prepare ozone demand free glassware and materials. Ozone gas generated from the ozone generator was bubbled through 4 L of Milli-Q water in an Erlenmeyer flask by means of a porous glass diffuser in fume hood for about 30 mins. The opening of the flask was covered with aluminum foil and the flask was left overnight to ensure that the aqueous ozone completely decomposed. The water was then considered ODF and was stored at room temperature in a 4 L brown glass bottle with a plastic cap.

ODF phosphate buffer (0.05 M, pH = 6.0) was used in the disinfection reactor and to prepare ozone stock solutions. This buffer was prepared by dissolving appropriate amounts of Na_2HPO_4 (2.36g) and KH_2PO_4 (4.53g) in 1000 mL ODF water. After dissolving, the pH was adjusted to 6.0 using 1 N NaOH or 1 N HCl. The phosphate

buffer was made ODF using the same methods described in the previous paragraph for ODF water.

3.6.2.3 Ozone demand free glassware

All apparatuses and materials used in the ozone and monochloramine disinfection experiments were treated to be ozone demand-free prior to experiments. Concentrated ozone solution at room temperature was produced by bubbling ozone gas through a 4 L flask of Milli-Q water for 30 mins. All materials that came into contact with disinfectants, such as reactor flasks, stir bars, glass pipettes, pipettes tips, 40 mL ozone sample vials, volumetric flasks, were then filled completely with the ozone solution, covered with aluminum foil and left overnight. The next day, the water was poured off and the materials were dried in an air oven at 121°C until dry.

3.6.2.4 Determination of dissolved ozone concentration

The direct UV absorbance (at 260 nm) and the indigo trisulphonate method (at 600 nm) were both used to measure the dissolved ozone concentration of ozone stock solution and aqueous ozone residual in the reactor. The indigo trisulphonate and direct UV absorbance methods are based on different principles. For the direct UV measurement, a 10 mm quartz cuvette was first filled with ODF phosphate buffer (pH= 6.0) and placed in a UV spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Cambridge, UK) to set a reference. The cuvette was then rinsed and filled with a fresh sample of aqueous ozone solution and quickly placed into the spectrophotometer. The

absorbance at 260 nm was recorded. The ozone concentration of the sample was determined from the following equation:

$$C = \left(A_{260,sample} - A_{260,reference}\right) \times 14.54 \qquad \text{Equation 3-2}$$

This method was used mainly to measure the concentration of ozone in the stock solution because it is rapid and is useful when there is little background absorbance. Ozone has molar extinction coefficient of 2,900 to 3,300$M^{-1}cm^{-1}$ at 258 to 260 nm in this equation (Hart, *et al.*, 1983). In this experiment, 3,300$M^{-1}cm^{-1}$ at 260 nm was used, and the concentration, therefore, can be determined by the difference in absorbance between a blank and the sample being measured. In equation 3-2, the molecular weight of 1 mole ozone is 48 g/mole, cuvette path length is 1 cm, so 1 mole ozone concentration = 48 g/mole / 3300 L/mole x 1000 = 14.54 mg/L .

To measure ozone residual in the disinfection reactor, where the background absorbance may have been higher, the indigo trisulphonate method was employed according to Standard Method 4500-O_3 B (Eaton, 1995) with some modification. A concentrated indigo stock solution was prepared by dissolving 0.770g potassium indigo trisulphonate (Sigma, Lot No: 117H3635) and 1 mL concentrated phosphate acid (Fisher Science) into ODF water to 1L. Indigo reagent II solution was produced by dissolving 100 mL of the prepared indigo stock solution, 10 g NaH_2PO_4 and 7 mL concentrated phosphorus acid into ODF water up to 1 L. To prepare a blank solution, 5 mL indigo reagent II solution and 15 mL pH 6.0 ODF phosphorus buffer were transferred into a 40 mL ODF vial with an ODF pipette. For experimental samples, 5 mL of indigo reagent II solution and 10 mL of pH 6.0 ODF phosphorus buffer were transferred into a 40 mL ODF vial. A 5 mL aliquot of the sample was slowly withdrawn from the ozone reactor

from a point near mid-depth of the reactor using an Oxford Macroset pipette (Oxford Labware, St. Louis, MO) fitted with a ODF plastic tip. The sample was quickly transferred to the vial. The absorbance of the sample and the blank was measured at 600 nm using an Ultrospec 2000 UV spectrophotometer and a 10 mm quartz cell. The spectrophotometer reference was set with ODF pH 6 phosphate buffer. Aqueous ozone concentration, in mg/L, was calculated according to the following equation:

$$C = \frac{(A_{600,Blank} - A_{600,Sample}) \times V_{Total}}{b \times f \times V_{Sample}} \qquad \text{Equation 3-3}$$

where:

$A_{600,Blank}$ = the absorbance of the blank at 600 nm in a 10 mm cell

$A_{600,Sample}$ = the absorbance of the sample at 600 nm in a 10 mm cell

V_{Total} = total volume of the solution in the vial (mL)

V_{Sample} = the volume of sample taken from reactor (mL)

b = the length of the cell (1cm)

f = sensitivity coefficient, 0.42 L/(mg x cm)

The factor $f = 0.42$ is based on a sensitivity factor of 20,000/cm for the change of absorbance (600nm) per mole of added ozone per liter.

3.6.2.5 Ozone disinfection system

The ozone disinfection system is illustrated in Figure 3-4. The ozone reactor (Reactor I) was a 500 mL Pyrex glass Erlenmeyer vacuum flask, fitted with an aspirating nozzle on the side of the neck. To start the experiments, the reactor was filled to the nozzle with ODF pH 6.0 phosphate buffer. A cement coupon with biofilm growth was

pretreated as described in section 3.4.1. The biofilm growth on one half (either top or bottom) of the coupon was removing by brushing and rinsing and the removed biofilm HPC bacteria were dispersed and enumerated as described in section 3.4.2. The concentration of bacteria from this half of the coupon was designated N_0 in Equation 2-2. The rest of the coupon was placed into the reactor to be disinfected.

Concentrated ozone stock solution was prepared as described in section 3.6.4.1 and was pumped from the 500 mL wash bottle into a disinfection reactor using a peristaltic pump (Model 7518-10 Cole Parmer, Vernon Hills, Illinois, USA) fitted with a number 13 pump head and number 13 black Norprene tubing. Because aqueous ozone is fairly unstable at room temperature, the contents of the wash bottle were kept cool by placing the wash bottle in an 8.5 L insulated plastic cooler that was filled with an ice-water mixture. At the same time that the pumping of the ozone stock solution pumped into the reactor was started, an aliquot of the same ozone stock solution was added directly to the reactor to quickly raise the concentration of the ozone to near the target value (1.0 mg/L), This was the start of the disinfection experiment (time zero). Ozone tends to decay spontaneously in solution; therefore, the ozone stock solution was pumped into the reactor at rate sufficient to maintain the ozone residual concentration constant at the target value. As the ozone solution was pumped into the reactor, the excess liquid overflowed through the nozzle via a plastic tube and was collected into a covered container that was filled with sodium formate solution to quench the residual aqueous ozone concentration. Therefore, a constant level and volume was maintained in the flask.

The reactor was placed in an insulated plastic cooler that was filled with water. To maintain the temperature of the reactor contents at 20°C, the water in the cooler was

circulated through a temperature-controlled water bath, set to 22°C (Model 1187P, VWR International, Bristol). The water in the cooler was recirculated within the cooler using a small aquarium pump to ensure uniform temperature. The temperature of the water in the reactor was recorded with a digital thermometer fitted with a probe (Traceable, Suzhou China). The reactor was placed on a stir plate (Isotemp, Fisher Scientific) and mixing was provided via an ODF Teflon-coated stir bar to ensure a uniform ozone concentration in the aqueous phase in the reactor. The reactor was assumed to behave as a continuous-stirred tank reactor with respect to the liquid and the biofilm on the coupon was assumed to be exposed to a spatially uniform aqueous ozone concentration that was approximately constant with time.

In order to provide a continuous and instantaneous measure of aqueous ozone concentration in the reactor for monitoring and control purposes, the reactor liquid was circulated through a UV-vis dual beam spectrophotometer (Model UV – 2401 PC, Shimadzu, Suzhou, China) that was equipped with an 80 μL quartz flow cell. The reactor liquid was pumped through the flow cell by means of a small peristaltic pump (Model Minipuls 3, Mandel, Guelph, Ont.). The absorbance of the reactor liquid was monitored in real-time using the flow cell. In this system, two quartz cells were needed for this UV-vis dual beam spectrophotometer. One conventional 10 mm quartz cell was filled with pH 6.0 ODF phosphate buffer and was placed in the reference cell holder. The 80 μL quartz flow cell was placed into the flow cell holder. A kinetic computer control program -- Spectro was used to monitor the absorbance in the flow cell. According to the direct UV absorbance (at 260 nm) method mentioned in 3.6.2.4, the wavelength of spectrophotometer was set to 260 nm. The target absorbance (0.069) was calculated by

dividing the known target ozone concentration (1.0 mg/L) by 14.45. The absorbance curve on the computer screen showed the on-line absorbance in the reactor. The actual measured absorbance was maintained close to the target absorbance by adjusting the flow rate of the ozone stock solution into the reactor.

Immediately after the ozone flow was started and the aliquot of ozone was added (i.e. time zero), a 5 mL sample was removed from the reactor and the ozone concentration was measured using the Indigo method (see 3.6.2.4). This was repeated every 5 or 10 minutes thereafter. Measurement of the ozone concentration in these grab samples using the indigo method was use to provide a more accurate assessment of the ozone residual concentration in the reactor (See Appendix A-10). When the desired ozone exposure time was reached, the ozone was neutralized by addition of sodium formate, and the coupon was removed from the reactor. The biofilm remaining on the coupon was removed using the brushing procedure and the HPC bacteria were dispersed and enumerated as described earlier. The concentration of HPC of this half of the coupon was designated as N in Equation 2-2.

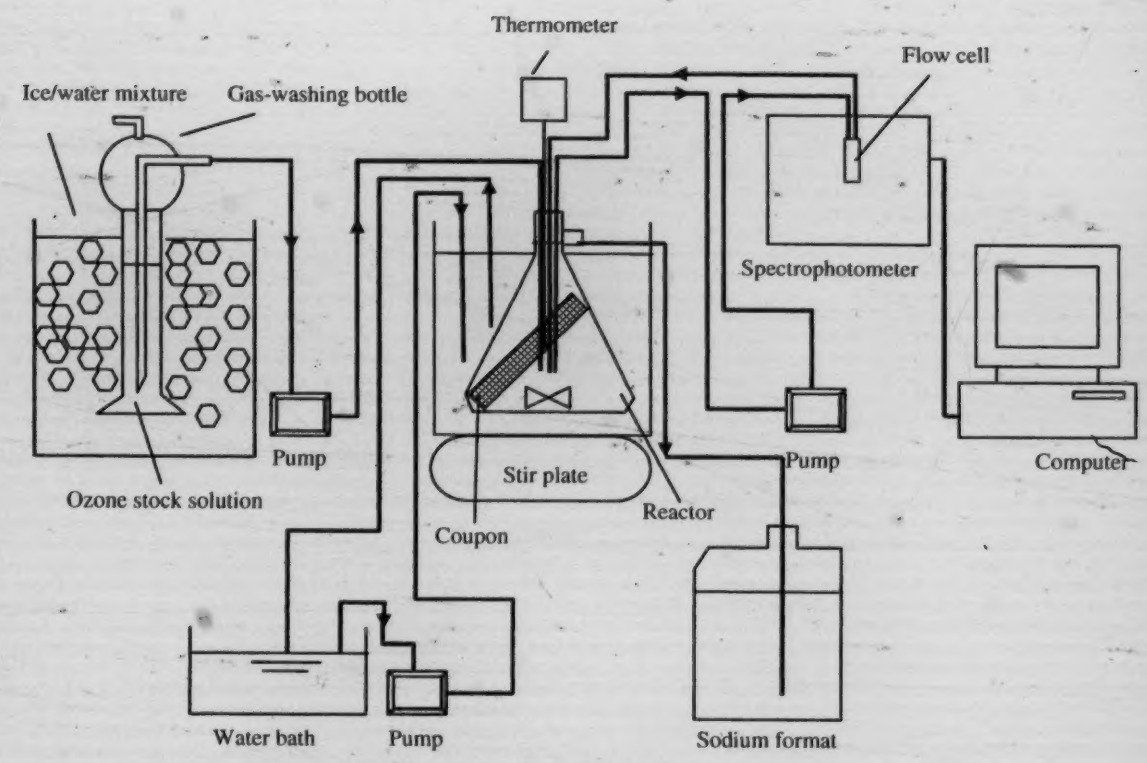

Figure 3-4 Schematic of the ozone biofilm disinfection apparatus

3.6.2.6 Determination of desired ozone exposure

As described in Section 3.6.2.5, the actual ozone residual was measured using the indigo method at 5 or 10 mins intervals during the disinfection process. The ozone concentration C_i (mg/L) was recorded at each sampling time t_i (min). An integrated average concentation –time product, $C_{avg}.t$ (mg x min/L), was calculated using the following equation:

$$C_{avg}t_i = \sum \frac{1}{2}(t_i - t_{i-1})(C_i + C_{i-1}) \qquad \text{Equation 3-4}$$

Where:

C_{avg} = Average ozone residual in the reactor during time $t = i - 1$ to $t = i$

t_i = time equal i

t_{i-1} = time equal i-1

C_i = Ozone residual in the reactor at t_i

C_{i-1} = Ozone residual in the reactor at t_{i-1}

Thus the accumulated $C_{avg}t$ was determined. When the total $C_{avg}t$ value equaled to the target $C_{av}t$ value, the disinfection experiment was terminated by adding excess sodium formate to quench the ozone residual in the reactor.

3.6.3 Disinfection with monochloramine

3.6.3.1 ODF phosphate buffer

ODF phosphate buffer at pH 7.5 was used in the monochloramine disinfection reactor (reactor II). This solution was prepared by dissolving appropriate amounts of Na_2HPO_4 (1.78 g) and NaH_2PO_4 (2.5 g) in ODF water with stirring to make 1 L of phosphate buffer. After dissolving completely, the pH was adjusted to 7.5 using 1 N NaOH. The concentration of the solution is 0.05M.

3.6.3.2 Monochloramine stock solution preparation

A 500 mg/L free chlorine stock solution was prepared by adding 10 mL of 5.25% sodium hypochlorite (Fisher Scientific, Lot No: 036259-9, Fair Lawn, New Jersey) to a 1000 mL volumetric flask and filling with Milli-Q water. Monochloramine solution was prepared based on the method described by LeChevallier et al. (1987). Ten mL of free chlorine stock solution and 6 mg of NH_4Cl were added to a 1000 mL ODF volumetric flask and the flask was filled with pH 7.5 ODF phosphate buffer to make a monochloramine solution with a concentration of 5.0 mg/L as available chlorine. The ratio of chlorine to nitrogen was 3 mg Cl_2:1 mg N. This stock solution was subsequently diluted to a 2.5 mg/L monochloramine solution using pH 7.5 ODF phosphate buffer solution.

3.6.3.3 Determination of monochloramine residual

The concentration of the total available chlorine was measured using the N,N-diethyl-p-phenylenediamine (DPD) colorimetric Standard Method 4500-Cl G (Eaton, 1995) with a minor modification. A 25 mL sample was removed from the reactor using an Oxford Macroset Pipette fitted with an ODF tip and was transferred into a 40 mL ODF glass vial. Total chlorine DPD reagent powder (for 25 mL volume, Lot No: A0252, HACH Co., Loveland, CO) was added immediately to the vial. The vial was capped and inverted several times until the DPD powder was completely dissolved. The absorbance at 515 nm was measured using an Ultrospec 2000 UV spectrophotometer and a 10 mm quartz cuvette. ODF buffer (pH 7.5) was used as a reference. The whole procedure was finished within 3 minutes. The DPD Ferrous Titration Method (Standard method 4500-Cl

F, Eaton *et al.* 1995) was used to verify that the total chlorine in the stock solution was comprised of at least 95% monochloramine. Therefore, it can be assumed that the total available chlorine was mostly comprised of monochloramine during the disinfection experiments.

3.6.3.4 Monochloramine disinfection system

A photograph of the monochloramine disinfection reactor is shown in Figure 3-5. Microorganism inactivation experiments with monochloramine were carried out in a well-mixed batch reactor (an ODF 1000 mL Pyrex Erlenmeyer flask). All experiments were carried out at 20°C ambient laboratory temperature (controlled by the thermostat in the lab). The solution in the reactor was mixed by means of an ODF Teflon-coated magnetic stir bar and magnetic stirrer set at a low mixing speed. At the start of a disinfection experiment, 1000 mL of a 2 to 2.5 mg/L monochloramine solution was added to the reactor. A biofilm coupon was removed from the bioreactor and scraped using a rubber policeman and rinsed as described previously (Section 3.4.1). The biofilm from either the top or bottom half was removed using the brush, and the HPC bacteria were dispersed and enumerated as described previously to determine N_0 (Section 3.4.2). The coupon was then placed into the reactor, and the biofilm submerged in the 2 to 2.5 mg/L monochloramine solution at time zero. The monochloramine concentration and the temperature were measured at the beginning, and after 6 and 24 hours. Experiments were terminated at 6 or 24 hrs by adding excess sodium formate. After a desired monochloramine exposure, the coupon was removed from the reactor. The biofilm remaining on the coupon was removed by brush and the HPC bacteria were dispersed and enumerated as described in Section 3.4.2 to determine N.

Figure 3-5 A monochloramine disinfection system

3.6.4 Disinfection with ozone combined with monochloramine

This process involved the sequential combination of ozone and monochloramine disinfection processes described before. For the sequential disinfection experiments, two disinfection reactors were employed, one of which was used for ozone disinfection (reactor I), and the other for monochloramine disinfection (reactor II). A coupon was scraped and rinsed as described in Section 3.4.1. Biofilm HPC was removed from one half of the coupon, dispersed and enumerated as described in Section 3.4.2 to determine N_0. The other half was placed in reactor I and exposed to aqueous ozone at the target ozone residual concentration for the desired time. When the desired ozone exposure time

was reached, the ozone was neutralized by addition of sodium formate. The ozonized coupon was removed from the reactor and was placed into reactor II. After the desired monochloramine exposure in reactor II, the coupon was removed and the HPC bacteria were removed and enumerated as described in Section 3.4.2 to determine *N*.

3.7 Quality control

A quality control process was used throughout the process of the study. A test strip was used to check sterilization efficacy of the autoclave once a week. The temperatures of autoclaves and incubators were monitored regularly to ensure they were in proper operating condition. The analytical balance, pH meter, turbidity meter, and DO meters were calibrated on a regular basis. The bench table top was sterilized using 70% alcohol or 30% bleach before and after bio-experiments. The HPC bacteria in blank dilution bottles (negative controls) were determined routinely during HPC bacteria enumerations to test for asceptic (sterile) technique.

3.8 Data analysis

All data were input into MS Excel™ 2003 spreadsheets. All statistical analyses and linear regression analyses were carried using the Analysis Toolpak in MS Excel. The Solver tool in MS Excel was used to carry out non-linear regression using least-squares criteria for fitting ozone inactivation models to the data.

4 Result and Discussion

4.1 Original water sample quality analysis

Pipe wash water from the Denver distribution system pipe storage yard was used in this experiment to cultivate biofilms. Thus, it was expected that the biofilm growing on cement coupons in the laboratory would be representative of the natural mixture of species growing on the interior walls of new pipe that was used to repair the Denver water distribution system. The properties of the three pipe wash water samples used for biofilm growth on cement coupons are shown below.

Table 4-1 Denver Water pipe wash water sample properties

Date	Sample	pH	Total chlorine (mg Cl_2/L)	Turbidity (NTU)	COD (mg O_2/L)	HPC bacteria (CFU/100ml)
Oct.12/05	1	6.13	0.001	45.5	778	6.2×10^8
Feb.14/06	2	6.72	0.028	5.33	77.0	6.8×10^8
Mar.24/06	3	6.21	0.187	42	1990	N/A

Note: N/A means not measured

As shown in Table 4-1, the pH range was between 6 and 7, which is suitable for HPC bacteria growth (HPC bacteria grow usually at pH 6 to 9). Very low (free or combined) chlorine residuals were detected in these samples. Therefore, a proper environment existed to ensure bacteria growth during biofilm cultivation. It was reasonable to observe a significant variation of turbidity between samples of pipe wash water because turbidity in wash water was largely dependent on the time when samples were taken and the conditions of pipe washing. The turbidity is normally high at the

early stage of flushing because of the washing out of dust and particles deposited in water main, while the turbidity decreases near the end of flushing.

In this study, COD was chosen to represent the concentration of organic substrate utilized by the biofilm bacteria in the biofilm reactor. Based on the initial COD, pipe wash water samples were mixed with buffer solution and added into the biofilm reactor during Phase I and Phase II experiments in order to maintain an approximately constant concentration of organic compounds during biofilm growth.

The wash water was relatively rich in HPC bacteria (i.e. approximately 10^8 CFU/100mL) and was expected to provide enough bacteria to seed the biofilm reactor for biofilm cultivation.

4.2 Biofilm growth conditions

Two batches of biofilms were cultivated in two separate bioreactor runs for this research study. A 20 L sample of pipe wash water sample was provided by Denver Water at the beginning of the first bioreactor run (Sample 1 in Table 4-1). It was realized later that this volume was only enough for completion of the first bioreactor run cultivation experiments. Later, samples 2 and 3 were delivered for the second bioreactor cultivation experiment. Therefore, two distinct sets of biofilms were cultivated for use in this study using three different samples of pipe wash water. Although the objective was to maintain consistent biofilm culture conditions during the experiments, in practice the growth conditions varied with the properties of the pipe wash water samples that were supplied by Denver Water. This was a practical limitation of the experiments.

The detailed monitoring data for both bioreactor runs are provided in Appendix A-2 and are summarized in the following sections.

4.2.1 COD

The total COD values in the biofilm reactor were adjusted and controlled to some extent during the biofilm cultivation process by addition of pipe wash water samples. The COD of the first pipe wash water sample received was 778 mg O_2/L (Table 4-1). Therefore, the target COD in the first bioreactor run was initially set at 150 mg O_2/L. In comparison, the average COD in municipal primary treated wastewater ranges from 100 to 200 mg O_2/ L. Figure 4-1 shows the actual COD concentration as a function of time during the bioreactor run 1. The initial COD concentration, shortly after inoculation, was approximately 150 mg/L, but the measured COD in the reactor increased to more than 300 mg O_2/L very quickly and continued to increase for about two weeks. The COD then decreased gradually until the end of the experiment. To maintain relatively constant conditions for biofilm development, the target COD value was then adjusted to 300 mg O_2/L. Additional pipe wash water sample was added to the reactor when the COD decreased below this value. The increase in COD at the beginning of the experiment is difficult to explain, but it may have been due to the increased concentration of suspended bacteria in the recirculating liquid and an associated increase in endogenous respiration that was greater than the rate of consumption of dissolved organic substrates. Endogenous respiration results in oxygen demand according to the following equation.

$$C_5H_7NO_2 + 5O_2 \xrightarrow{\text{bacteria}} 5CO_2 + 2H_2O + NH_3 + \text{energy}$$

Here, $C_5H_7NO_2$ represents the approximate molecular concentration of the typical bacterial cell. Another potential explanation is that there was organic material in the

wash water that was not oxidizable by the COD test. This non-oxidizable material may have been broken down by extracellular enzymes into oxidizable material. A third explanation is that algae growth was occurring. Algae are able to fix inorganic carbon and convert it into organic carbon by photosynthesis. But this explanation is only reasonable in the first bioreactor run of the experiment because the bioreactor system was not covered during bacteria cultivation. The light could go through the reactor and reservoir and result in algae photosynthesis and growth. In the second run, the equipment was all covered by aluminum foil at all times and light was unable to get through. However, the same increase trend of COD was measured. That is hard to explain because the algae can not alive without photosynthesis. So in the second run, the first two reasons might be dominant.

In the second bioreactor run, the target COD concentration in the biofilm reactor was set at 50 mg O_2/L due to the lower COD concentration in the second pipe wash water sample provided by Denver Water. The COD of second pipe wash water sample was 77.4 mg O_2/L. This sample was diluted with phosphate buffer to adjust the COD to 50 mg O_2/L and the bioreactor was inoculated with this solution. As in the first bioreactor run, an increase in COD in the bioreactor recirculation water was observed at the beginning of experiment. The COD increased to around 100 mg O_2/L (See Figure 4-2). After about three weeks of biofilm cultivation, the COD decreased to less than 50 mg O_2/L. Additional pipe wash water sample was added at this time. When the second sample of pipe wash water was consumed at about 42th day, a third sample of pipe wash water was received from Denver Water. This third sample had a much higher COD of

1990 mg O_2/L. Based on visual observation of the coupon surfaces, it was noticed that biofilms grew at a much slower rate than in the first bioreactor run, perhaps due to the lower substrate concentration. The target COD, therefore, was increased to 150 mg O_2/L in order to accelerate biofilm growth at about the 90th day. Volumes of pipe wash water sample were fed into the reactor periodically to maintain the COD of the recirculating water to less than 150 mg O_2/L. Based on a visual observation, the biofilms developed very well after that.

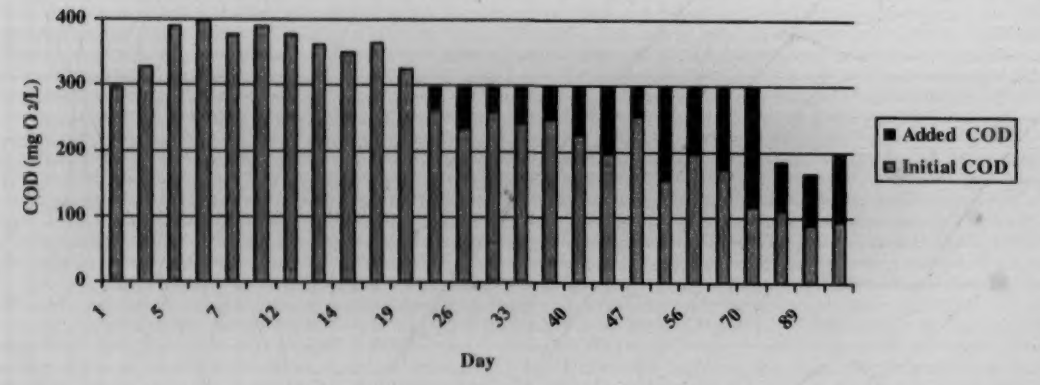

Figure 4-1 Measured COD (mg O_2/ L) in the recirculation water during the first bioreactor run.

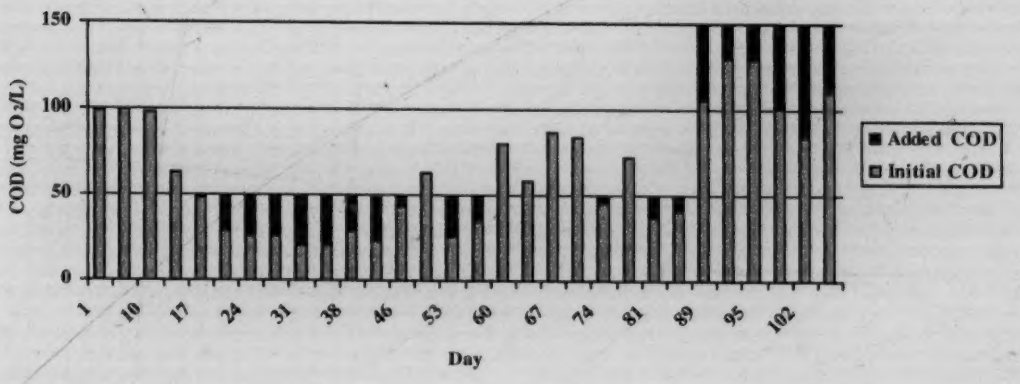

Figure 4-2 Measured COD (mg O_2/ L) in the recirculation water during the second bioreactor run.

4.2.2 pH

The recorded pH values of both biofilm cultivation experiments are illustrated in Figures 4-3 and 4-4, respectively.

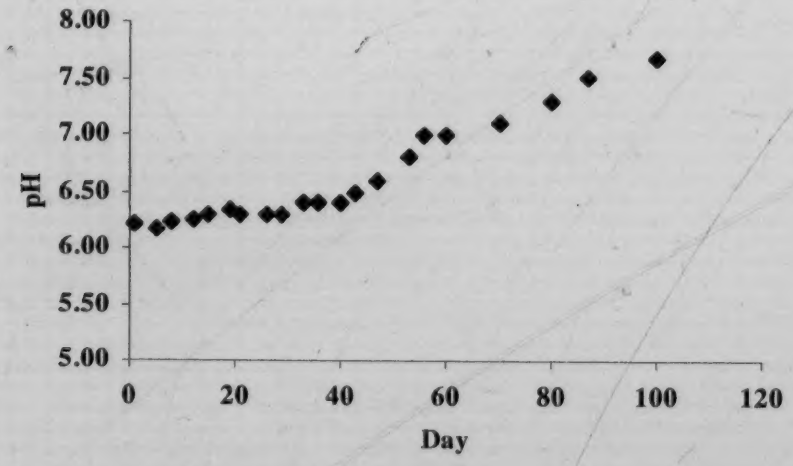

Figure 4-3 Monitored pH value for biofilm growth (First bioreactor run)

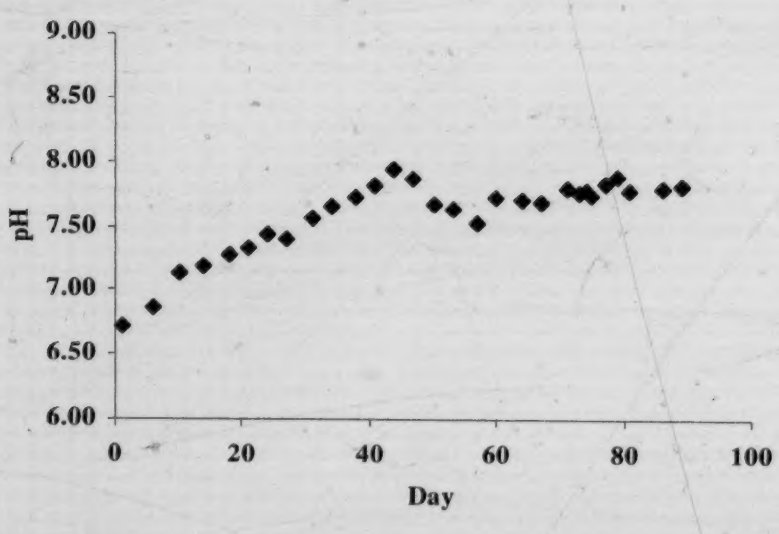

Figure 4-4 Monitored pH value for biofilm growth (Second bioreactor run)

The pH increased gradually in both biofilm reactor runs. In the first run, the pH increased from 6.17 to 7.69. In the second run, the pH increased from 6.60 to 7.82. It is unclear at this point why the pH increased in the biofilm reactor. Aerobic bacterial respiration produces carbon dioxide according to the following general formula:

$$\underset{\text{Organic matter}}{COHNS + O_2 + \text{nutrients}} \xrightarrow{\text{bacteria}} \underset{\text{New bacteria cell}}{CO_2 + NH_3 + C_5H_7NO_2} + \text{other end products}$$

Since carbon dioxide is a weak acid, a reduction in pH was expected.

The pH value in the second biofilm reactor run showed a similar increasing trend. A slight decrease was observed starting on the 42nd day, likely because of new batch of wash water with lower pH was added to the biofilm reactor. During the biofilm

cultivation period, water samples were routinely removed from the reactor recirculation tank for monitoring purposes. This resulted in a gradual decrease in the solution volume in the reactor. Also, the COD concentration decreased as it was consumed by the biofilm microorganisms. Fresh pipe wash water was added to the system to replace the lost volume and maintain a target COD concentration in the reactor. The COD of the first sample of wash water used in the first bioreactor run was 777 mg O_2/L and the target COD concentration of the bioreactor was 150 mg O_2/L. The COD of the second sample of wash water was considerably lower, at only 77.4 mg O_2/L. The initial target COD concentration in the second bioreactor run was, therefore, reduced to 50 mg O_2/L. Also, the wash water was added much more frequently during the second bioreactor run and was completely consumed at around the 42nd day of the experiment. The third sample of wash water was then delivered by Denver Water and was added to the biofilm reactor to complete the second bioreactor run. The pH of the third batch pipe wash water sample was less than the pH of the second one, resulting a decrease in the solution pH in the reactor.

4.2.3 Temperature

Figures 4-5 and 4-6 indicate the monitored temperatures of the recirculation water during both biofilm cultivation experiments.

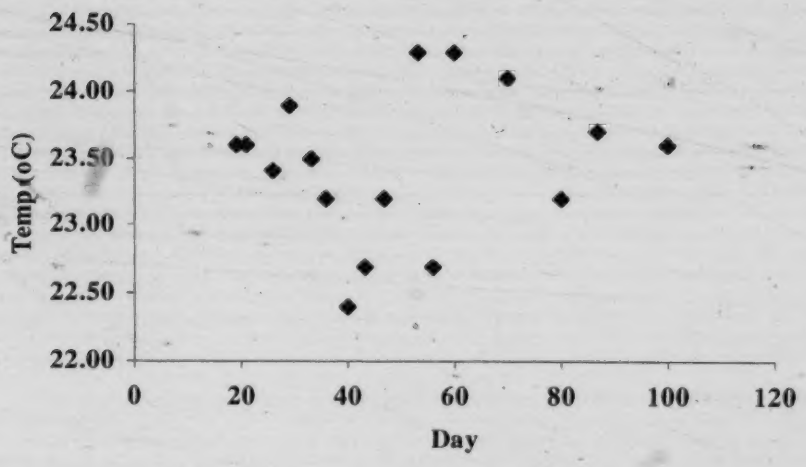

Figure 4-5 Recirculation water temperature (°C) during the first bioreactor run

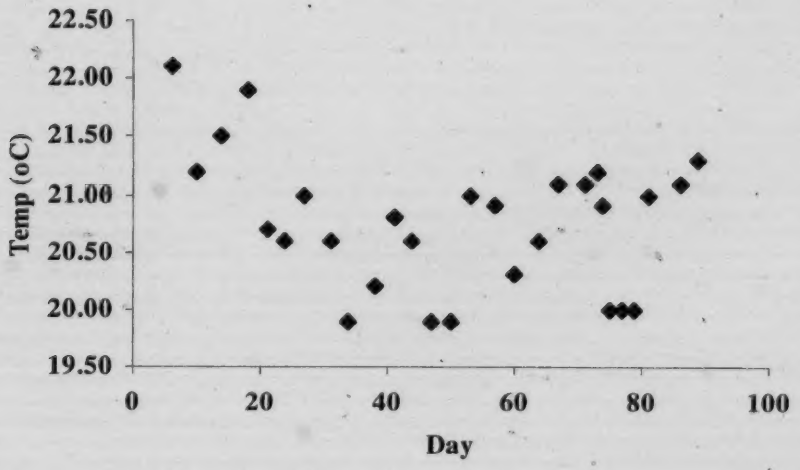

Figure 4-6 Recirculation water temperature (°C) during the second bioreactor run.

The biofilm growth temperature was determined mainly by the ambient temperature in the laboratory. The ambient temperature was controlled at 22°C during the first bioreactor run and the first 18 to 20 days of the second bioreactor run. After that,

the ambient temperature was controlled at 20.0°C. The temperature setpoint was determined somewhat by other experiments that were going in the lab. This is typical of a normal temperature for biofilm growth in summer. The recorded data showed that the temperatures in the biofilm reactor were a one or two degrees higher than this ambient lab temperature. The possible reasons include the heat generated from motor rotating and bacterial metabolic activity.

4.2.4 Dissolved Oxygen

The aerobic environment in the reactor was achieved by using diffused aeration of the water in the recirculation tank. Aeration also helped to ensure that the water in the tank was well mixed. Dissolved oxygen (DO) in the recirculation tank was measured twice a week and the monitoring results are shown in Figures 4-7 and 4-8 for and the first and second bioreactor runs, respectively. The DO was maintained between approximately 7 and 9 mg/L for Phase I and between 6 and 9 mg/L for Phase II biofilm experiments, respectively. In general, the dissolved oxygen concentration a wastewater treatment aeration tank should be maintained at 1.5 to 2.0 mg/L or greater so that oxygen is not growth limiting (Eddy, 2003). The measured DO value in the biofilm reactor, therefore, was likely sufficient to ensure that oxygen was not limiting, at least at the surface of the biofilms.

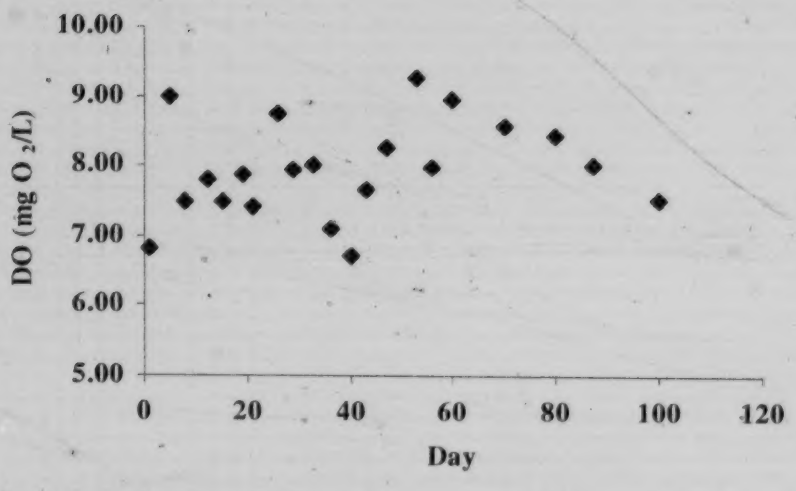

Figure 4-7 Measured DO (mg/L) concentration in the bioreactor re-circulation water for the first bioreactor run

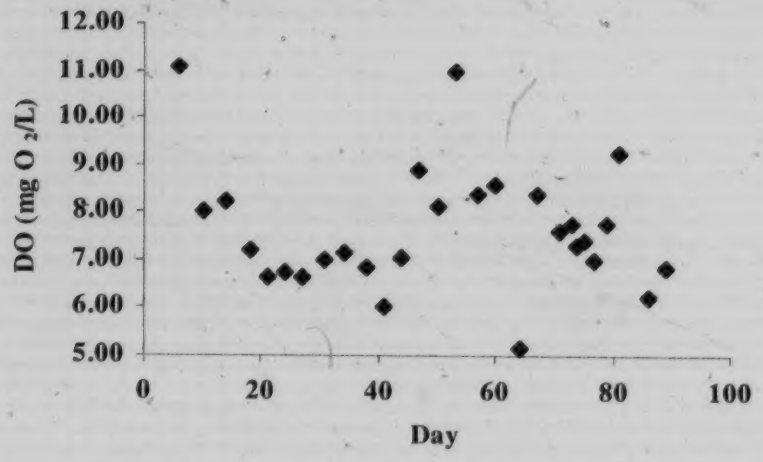

Figure 4-8 Measured DO (mg/L) concentration in the bioreactor recirculation water for the second bioreactor run.

4.2.5 Turbidity

Figure 4-9 illustrates the variation in turbidity in the bioreactor recirculating water during the first bioreactor run.

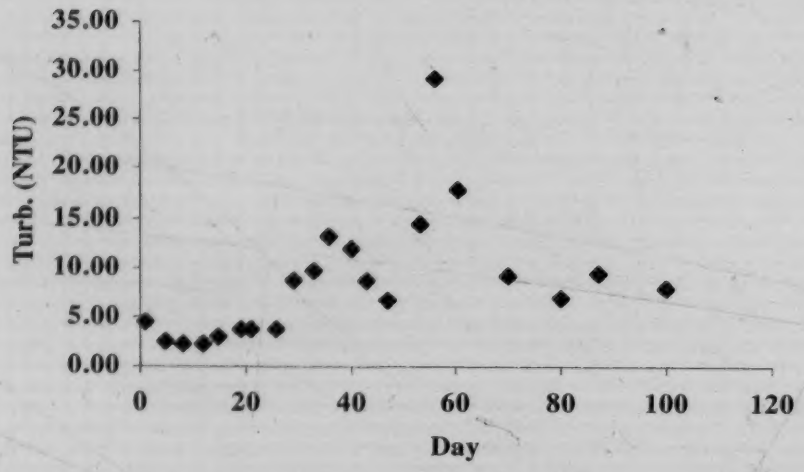

Figure 4-9 Measured turbidity (NTU) in the recirculation water during the first bioreactor run.

At the start of biofilm cultivation, when the pipe wash water-buffer solution was initially fed into the reactor, the turbidity was approximately 4.5 NTU. The turbidity decreased to 2.1 NTU after 10 days but then began to increase to as high as 29 NTU in the latter phase of the run. The initial decrease in turbidity may have been due to settling of larger particles in the feed water, or due to deposition and attachment of suspended bacteria onto the surface of the cement coupons and other internal surfaces of the reactor wall during the early period of biofilm cultivation. With gradual growth and maturation of the biofilms, some bacteria may have begun to detach (or slough) from the coupon and reactor surfaces, and return to the liquid phase. In addition, growth of suspended bacteria may have contributed to the increase in turbidity. The detached bacteria gradually formed larger flocs in the re-circulating water and then began to settle out at the bottom

of the reactor resulting in another decrease in turbidity during the latter part of the bioreactor run (i.e. after 60 days).

The variation in turbidity in the recirculation water of the second bioreactor run reactor experiment is shown in Figure 4-10.

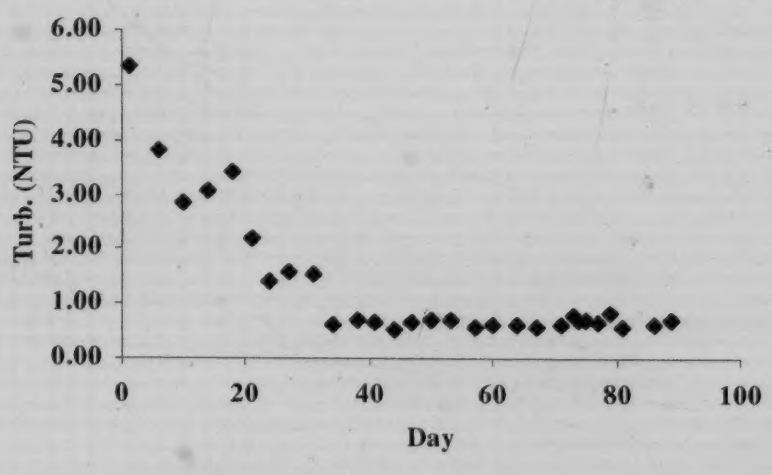

Figure 4-10 Measured turbidity (NTU) in the re-circulation water during the second bioreactor run

The turbidity in the second bioreactor run showed a more continuous decrease with time as compared to the first bioreactor run. The initial turbidity of the second bioreactor run was 5.25 NTU and decreased until the 10th day when the value was 2.9 NTU. Turbidity then increased slightly to 3.5 NTU and decreased again. The turbidity gradually reached a stable value of 0.6 NTU around the 40th day and remained to the end of the experiment. The difference in turbidity trends in the two experiments was possibly due to the differences in the pipe wash water samples used to inoculate the bioreactors. The water samples using in the second bioreactor run may have contained larger and

heavier particles that settled more readily. Another possible reason was the lower COD during the first part of the second bioreactor run. A lower organic substrate concentration may have resulted in slower biofilm and suspended bacterial growth. Due to slower, substrate-limited growth, the biofilm bacteria attached to the coupons and container surfaces may not have developed to the point where detachment from the surface was significant.

4.3 Testing the protocol for biofilm HPC bacteria enumeration

Before carrying out biofilm disinfection experiments, it was necessary to develop a procedure for effectively removing biofilm bacteria from the cement coupon surface and then enumerating the bacteria. In previous biofilm disinfection experiments (Mathrani, 2006), the biofilm was removed by simply scraping the cement surface with a metal spatula into a buffer solution. The solution was then homogenized using a tissue homogenizer and the HPC bacteria concentration was determined. Using this procedure, Mathrani (2006) observed unusual results when the biofilms grown on cement coupons in a similar biofilm reactor were exposed to disinfectants. After exposure to low doses of ozone and free chlorine, the measured number of bacteria on the cement surface was actually greater than before exposure to the chemical. These unusual results may have been due to the incomplete or inefficient removal and enumeration of biofilm bacteria. One of the objectives in this study was, therefore, to improve the techniques used for biofilm removal and enumeration.

In order to accurately enumerate biofilm bacteria, both physical and chemical elution processes were tested for their ability to remove bacteria from the cement surface

of the coupons and to disperse the bacteria in buffer solutions. In place of the metal spatula used by Mathrani (2006), a plastic bristle brush was used to scrub the surfaces of the cement coupons in order to remove bacteria attached within surface holes and cracks more effectively. Once the biofilm was removed from the coupon, the dispersion step was used to disrupt aggregates or clumps, and produce a monodispersed bacterial suspension. The assumption in HPC bacterial enumeration is that one colony counted on the analytical filter arises from a single bacterium. This is only true if the bacteria are monodispersed.

The physical approach was to use a tissue homogenizer to disrupt bacterial aggregates, or clumps, and to disperse bacteria in solution. The extent of dispersion was expected to be a function of homogenization time and intensity. Homogenization intensity was varied by adjusting the rotational speed of the homogenizer cutting tip. Two homogenizer speeds (13,500 rpm or 25,000 rpm) and three homogenization times (2, 6 and 10 minutes) were tested. In previous studies chemical solutions containing surfactants have been used to remove bacteria form surfaces such as activated carbon (Camper *et al.*, 1984), and to improve dispersal of bacteria. In this study, two solutions were tested and compared for their ability to aid in the removal of biofilm bacteria from the cement surfaces and dispersal in solution: 0.05 M pH 6.0 phosphate buffer solution and 0.01 M, pH 7.0 surfactant solution (Camper's solution - see Section 3.4.3 for the composition). A factorial design was set up to test the effects of homogenization time, intensity and dispersal solution. The factorial design and experimental results are shown in Table 4-2. For this experiment, coupons were extracted from the biofilm reactor (the first bioreactor run) and were exposed to the treatment combinations described in Table

4-2. Each experimental combination was tested twice using a different coupon. The experiment was done over a period of 4 weeks, and the runs were randomized. In Table 4-2, the HPC bacteria concentration is shown in both terms of the number of colony forming units (CFU) per mL of dispersal solution and in terms of CFU per square centimeter of cement surface area.

Table 4-2 Efficiency of different homogenization techniques for dispersion of HPC bacteria from biofilm

Homogenizer Speed	Dispersion Reagent	Time (min)	HPC Bacteria Concentration			
			Based on Eluting Solution Volume 10^6 CFU/100mL		Based on Coupon Surface Area 10^6 CFU/cm^2	
			Rep. 1	Rep. 2	Rep. 1	Rep. 2
13,500 rpm	0.05M pH 6.0 Phosphate Buffer	2	46	33	3.1	2.2
		6	30	N/A	2.0	N/A
		10	33	N/A	2.2	N/A
	0.01 M pH 7.0 Camper solution	2	TNTC	TNTC	TNTC	TNTC
		6	TNTC	TNTC	TNTC	TNTC
		10	TNTC	TNTC	TNTC	TNTC
25,000 rpm	0.05 M pH 6.0 Phosphate Buffer	2	32	37	2.1	2.5
		6	31	30	2.1	2
		10	31	33	2.1	2.2
	0.01 M pH 7.0 Camper solution	2	TNTC	TNTC	TNTC	TNTC
		6	TNTC	TNTC	TNTC	TNTC
		10	TNTC	TNTC	TNTC	TNTC

Notes: Rep. 1 and Rep.2 are the results of duplicate experiments at the same conditions

N/A = result not available due to contamination of the plates

TNTC = too numerous to count

4.3.1 Effect of dispersal solution

A quick inspection of the results in Table 4-2 shows that the use of the Camper's solution had a significant effect on the measured bacterial cell concentration. In all experiments in which the Camper's solution was used, even at the lowest homogenizer speed and shortest homogenization time, the concentration of HPC bacteria was too numerous to count (TNTC). Clearly, the Camper solution was much more effective at dispersing the bacteria than was the phosphate buffer. This also suggests that use of the phosphate buffer would result in significant underestimation of the concentration of HPC bacteria on the cement surface. One of the concerns with the use of the dispersal techniques, whether physical or chemical, is that the dispersal method may actually result in lower measured bacteria concentrations due to death of the cells if the method is too aggressive. For the Camper's solution, there was little evidence that this was occurring or perhaps the improved dispersal greatly outweighed any increased cell death due to exposure to the dispersal solution. In addition, the number of HPC bacteria was too numerous to count when the Camper's solution was used. Because of the beyond detection results, it was not possible to analyze the variability of the method. Moreover, this variability would affect the enumeration of HPC bacteria before and after disinfection and the evaluation of inactivation efficiency. This was identified as one of the limitations of the experiment. Nevertheless, it is clear that Camper's solution resulted

in a significant increase of enumeration of the bacteria compared with phosphate buffer solution.

4.3.2 Homogenizer speed and time effects on HPC for phosphate buffer method

In order to study the effect of homogenization time and intensity on HPC enumeration quantitatively, the phosphate buffer data set from Table 4-2 was examined using a linear regression approach with Microsoft Excel. Using only the phosphate buffer data from Table 4-2, a linear regression model of following form was tested:

$$\text{HPC } (10^6 \text{ CFU/100mL}) = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_{12} X_1 X_2 \qquad \text{Equation 4-1}$$

Where: X_1 represents homogenizer speed (rpm)

X_2 represents homogenizer time (minutes)

A regression analysis (details in Appendix A-4), indicated that none of the regressors (X_1, X_2 or the interaction X_1X_2) were statistically significant. That is, there was no statistical evidence that either homogenization intensity or time had an effect on the bacterial counts for the experimental ranges tested. Although the results suggest that the homogenization conditions had no effect on the bacterial counts for the experimental ranges tested, the result is still important because it provides evidence that the homogenization conditions were not adversely impacting the bacterial counts by killing or inactivating bacterial cells. If the homogenization resulted in cell inactivation, than

increasing the intensity or time or homogenization would have resulted in statistically significant decrease in colony counts. This was not observed.

4.3.3 Homogenizer speed and time effects on HPC for Camper's solution method

Neither homogenization intensity nor time had a significant effect on biofilm HPC bacteria when phosphate buffer was used as the dispersal solution. Since all the bacterial results were too numerous to count (TNTC) when the Camper's solution was used, it was not possible to test for the effect of homogenization intensity or time. However, it was assumed that the homogenization conditions would have a similar insignificant effect on biofilm HPC bacteria enumeration when the Camper buffer was used. Based on the results of the factorial experiment, the protocol for biofilm bacteria removal and dispersal summarized in Table 4-3 was chosen for subsequent disinfection experiments. The Camper's solution was selected because it provided the greatest removal and dispersal of HPC bacteria and that was the objective at the outset of experiments. Since homogenization time does not make any difference to the results, the shorter time was selected. In addition, the speed was set to 25,000 rpm to maximize dispersion of bacteria.

Table 4-3 Homogenization and dispersion efficiency experiment result – conditions chosen for further experiments

Homogenization condition		Dispersion Reagent
Speed (rpm)	Time (minutes)	
25,000	2	Camper's solution

The results of this study are consistent with the results reported by Camper *et al.* (1984). These researchers studied desorption of bacteria from GAC (granular activated carbon) in 19 different kinds of dispersal solutions. They found that homogenization at 16,000 rpm for 3 min at 4°C with a mixture of 0.01% peptone, 10^{-6} M Zwittergent 3-12, pH 7.0, 0.01M Tris buffer and 0.001M EGTA was the most effective for desorption of both HPC and *coliform* bacteria from GAC particles. Their results also showed that there was little difference between a mixture of 0.01% peptone, 10^{-6} M Zwittergent 3-12, pH 7.0, 0.01M Tris buffer and 0.001M EGTA and the mixture of 0.01% peptone, 10^{-6} M Zwittergent 3-12, pH 7.0, 0.01M Tris buffer (Camper's solution). Approximately 90% of the known bacterial population was recovered by plate count using the dispersion solutions. In the present study, at least four or five times the number of HPC bacteria was observed in the wash solution when Camper's solution was used in comparison to when the phosphate buffer was used as dispersal solution.

It should also be remembered that homogenization and dispersion techniques are not the only experimental variables that affect the recovery of bacteria from biofilms. The type of growth medium and incubation conditions used for enumeration may also play a role in determining the final bacterial counts. The results of Geldreich (1990) indicated that R2A agar yields significantly higher bacterial counts than Plant Count Agar (PCA) . McFeters (1990) reported the longer the incubation, the higher the viable HPC bacterial count when he used 3 to 7 days as incubation time at 20°C to 37°C for enumeration of HPC bacteria. The HPC data of Reasoner and Geldreich (1985) illustrates the effect of incubation temperature on HPC results. The measured concentration of HPC bacteria in drinking water using on any growth medium decrease as the incubation temperature was

increased from 20°C to 28°C to 35°C. In this study, a fixed protocol was used for enumerating the HPC bacteria. The bacteria were captured on a 0.45 μm pore size filter and were incubated at 20°C for 7 days on R2A agar because the bacteria grown on the interior surface of the pipe which are put outside field where temperature is around 20 °C at the summer. The temperature of 20 °C during the incubation is more appropriate for this bacteria species growing in the real pipe in a pipe yard.

It should be kept in mind that, even using vigorous physical removal and dispersal procedures like those used in this study; it is probably not possible to completely remove all bacteria from a rough surface like cement and to completely disperse the removed biofilm bacteria in solution. Also, many bacteria in water will be viable but non-culturable. Culture methods will only capture a relatively small proportion of the bacteria that are present (Bartram *et al.*, 2003a). However, given these unavoidable experimental limitations, it is important to attempt to improve the quantitative removal and dispersal of bacteria and to arrive at a consistent method that can be used in disinfection studies. In this study, a removal and dispersal method was developed that provides solid foundation for subsequent disinfection experiments.

4.4 Biofilm Inactivation Experiments

4.4.1 Disinfection of Biofilms Using Ozone

Biofilms from the second biofilm cultivation experiment were exposed to ozone using the continuous-flow ozone reactor apparatus described in Figure 3-4. The target aqueous ozone residual concentration in the experimental ozone reactor was 1.0 mg/L. The ozone residual was monitored continuously by the flow-cell spectrophotometer and

computer program, and the ozone addition rate was adjusted in order to maintain the ozone residual at the target. The actual measured ozone residuals measured varied between 0.5 and 3 mg/L during the experiments. Ozone residuals were confirmed by periodic sampling and analysis using the indigo trisulphonate method. Figure 4-11 shows the ozone residual concentrations measured using the Indigo method and the on-line absorbance measured as a function of exposure time for a typical ozone disinfection experiment. In this experiment, the actual ozone residual concentration (Indigo method) varied between 0.75 and 1.5 mg/L and the average concentration, C_{avg}, was 1.04 mg/L. The ozone exposure period, t, was 120 minutes and the integrated $C_{avg}t$ product was 123.7 mg x min/L. Measured ozone concentration (on-line absorbance method) was between 0.29 and 0.72 mg/ L from Figure 4-11. Although both methods produced different numerical values, the patterns revealed were the same. Peak values and bottom values appeared at the same time. Also, the relative difference between the two values was relatively constant. Two exceptional on-line measurement values were observed at 25 and 85 min (Figure 4-11). These were likely caused by a concentrated ozone bubble that entered the flow cell when the absorbance was record. Because the volume of the flow cell was small (80 μL), an ozone bubble can make large impact on the measured result. But beside these two points, the rest of the measurements are in a reasonable range. Similar ozone data for the other disinfection experiments is provided in Appendix A-11. The $C_{avg}t$ products for the other ozone biofilm disinfection experiments were calculated in a similar way using the data in Appendix A-11.

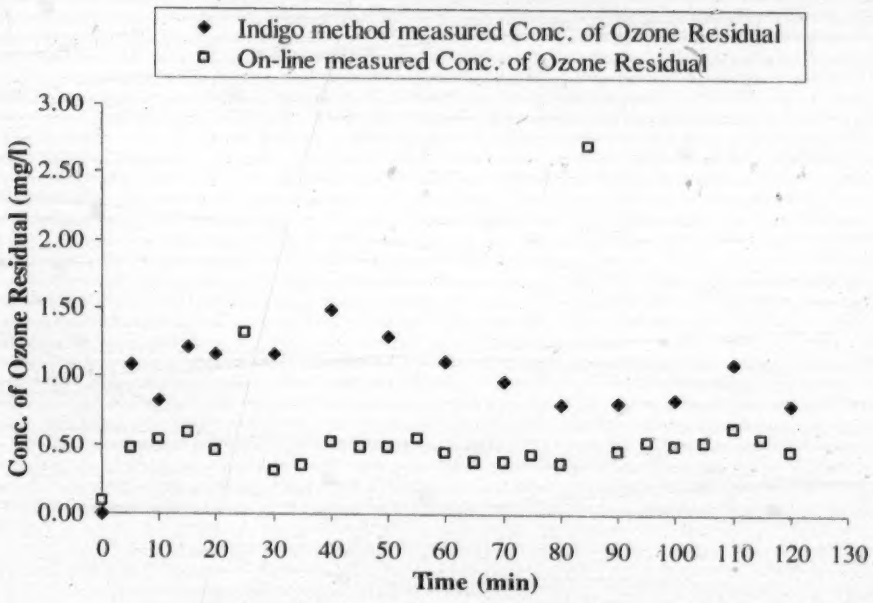

Figure 4-11 Measured ozone residual in a typical ozone experiment. The contact time was 120 minutes and the integrated ozone $C_{avg}t$ product was 123.7 mg x min/L (Indigo method). Ozone addition was started at time zero and ozone residual was neutralized at 120 minutes.

Four target ozone $C_{avg}t$ products were investigated (10, 60, 120 and 240 mg x min/L) in the ozone biofilm disinfection experiments. Each target $C_{avg}t$ was tested in duplicate except the value of 240 mg x min/L which was tested only once. The results of the biofilm disinfection experiments are summarized in Table 4-4. The experiments were done in random order as indicated in the table.

Table 4-4 Results of biofilm inactivation experiments using ozone. All experiments were carried out 0.05 M pH 6.0 ODF phosphate buffer at 22°C.

Run No.	Avg. Ozone Residual, C_{avg} (mg/L)	Contact time, t (min.)	$C_{avg}t$ (mg x min/L)	Inactivation Log (N_0/N)
6	1.076	15	13.4	0.28
8	0.911	15	11.3	0.61
5	0.965	60	56.5	0.61
3	2.649	33	78.1	1.65
7	1.132	120	138	2.17
4	1.144	110	126	1.52
1	1.237	100	123	1.68
2	2.05	120	236	1.71

Note: Average. ozone residual values, C_{avg} (mg/L), are based on the indigo method measurements

Table 4-4 shows that HPC bacteria inactivation increase with increasing $C_{avg}t$ values. The inactivation effect increased from 0.2 to 1.68 log when $C_{avg}t$ increased from 11.3 to 123 mg x min/L. When the $C_{avg}t$ value increased further from 123 to 236 mg x min/L, no significant change was observed in biofilm HPC bacteria inactivation. The highest inactivation (2.1 log) occurred at $C_{avg}t$ equal to 138 mg x min/L. Inactivation results at all other $C_{avg}t$ values were less than 2 log. The data suggests a strong tailing effect where little further inactivation is achieved at increasing ozone exposures. Because only one experiment was conducted at $C_{av}t$ of 240 mg x min/L, it was not possible to conclude that the inactivation effect would not increase with higher ozone $C_{avg}t$ values.

More experiments with $C_{avg}t$ value greater than 120 mg x min/L should be conducted in future research to confirm the extent of the tailing effect.

Compared to the results reported by Viera et al. (1993), the efficacy of ozone for inactivation of biofilm bacteria observed in this study was lower. When they applied 0.5 mg/L ozone for 10 minutes to inactivate biofilms on stainless steel and carbon steel surfaces (i.e. $C_{avg}t$ of 5 mg x min/L), the number of bacteria was reduced by 2 log. In comparison, inactivation of HPC bacteria grown on cement surfaces in this study was less than 1 log at ozone $C_{avg}t$ values greater than 50 mg x min/L and was still less than 2 log even at a $C_{avg}t$ of 120 mg x min/L. One possible reason for the different biofilm inactivation rates achieved between the two studies might be the different surfaces on which the biofilms were grown. The biofilms grown on the stainless steel and carbon steel coupons may have been more susceptible to ozone than those grown on the cement coupon. The finding that different pipe materials could impact disinfection efficiency of attached bacteria has been reported by several researchers (LeChevallier *et al.*, 1990, Clark *et al.*, 1994, Momba and Makala, 2004). No reports have indicated that ozone can achieve more than 2 log inactivation of biofilm bacteria any surface.

4.4.2 Ozone inactivation model development

An essential feature of a disinfection kinetic model is simplification and idealization of a complex phenomenon. The Chick-Watson law and the Hom law were applied to develop models to express the rate of biofilm inactivation as function of the aqueous ozone concentration in the bulk liquid. These models were chosen because they have been used previously to model microorganism inactivation for water and wastewater

treatment processes. For example, Gyürék et al. (1997) used these models to described suspended HPC bacteria inactivation by ozone.

In order to test the kinetic models, biofilm HPC bacteria inactivation, expressed in logarithms of the inverse survival ratio (log N_0/N), were plotted as a function of cumulative dissolved ozone $C_{avg}t$. The experimental data are compared to least-squares best fits of both Chick-Watson model and Hom models in Figure 4-12 and 4-13, respectively. Non-linear least-squares calculations were performed using the Solver tool in a Microsoft Excel™ 2003 spreadsheet. The best fit equations are provided beneath each figure.

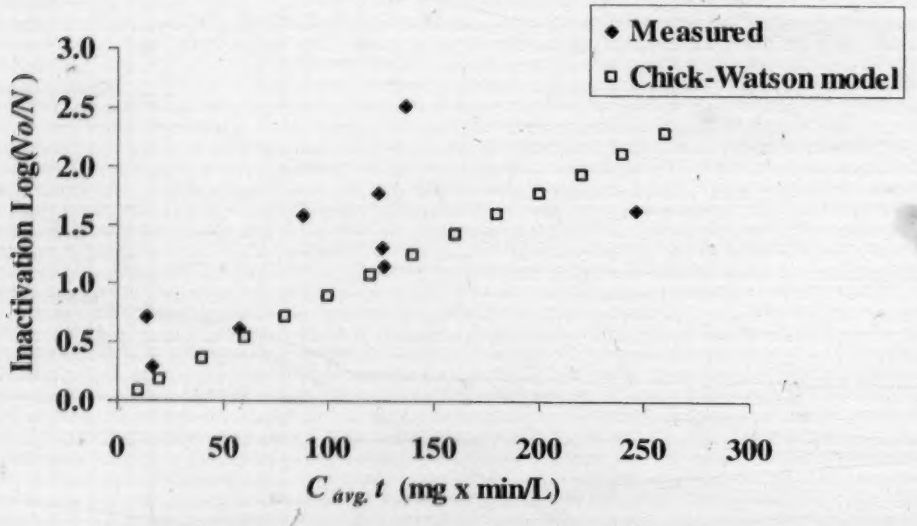

Figure 4-12 Comparison between measured biofilm HPC bacteria inactivation by ozone and that predicted using the Chick-Watson kinetic models

Chick-Watson: $\log\frac{N_0}{N} = 0.014C_{avg}^{\ 0.335}t$ Equation 4-1

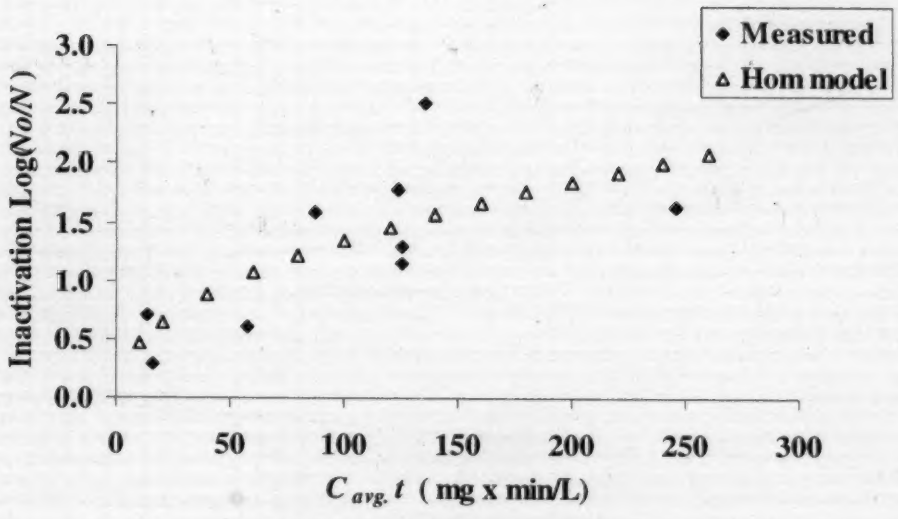

Figure 4-13 Comparison between measured biofilm HPC bacteria inactivation by ozone and that predicted using Hom Kinetic models

Hom model: $$\log\frac{N_0}{N} = 0.169 C_{avg}^{\ 0.426} t^{0.456}$$ Equation 4-2

As shown in Figure 4-13, the measured inactivation increased with increasing $C_{avg}t$, however the relationship between inactivation and $C_{avg}t$ was not first-order. Residual plots for the Chick-Watson and Hom model are provided in Figure 4-14 and 4-15, respectively. The residuals of the Chick-Watson were not evenly distributed around the zero and the measured inactivation exceeded the predicted values more often than not. The Chick-Watson model, therefore, did not fit the measured data very well. In

comparison, the fit of the Hom model predictions to the data was better and the model residuals were more evenly scattered around zero. The error sum of square of the Hom model (1.77) was smaller than that of the Chick-Watson model (2.51), which suggests that the predicted values of Hom model fit the experimental data better also. However, this is balanced somewhat by the fact that the Hom model has 3 adjustable parameters (*k*, *m* and *n*) while the Chick-Watson model has only two (*k* and *m*)

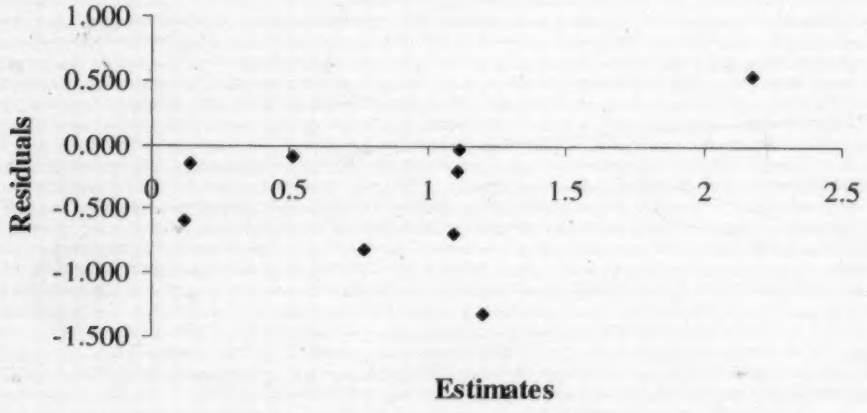

Figure 4-14 Residual – Estimate plot of Chick - Watson model

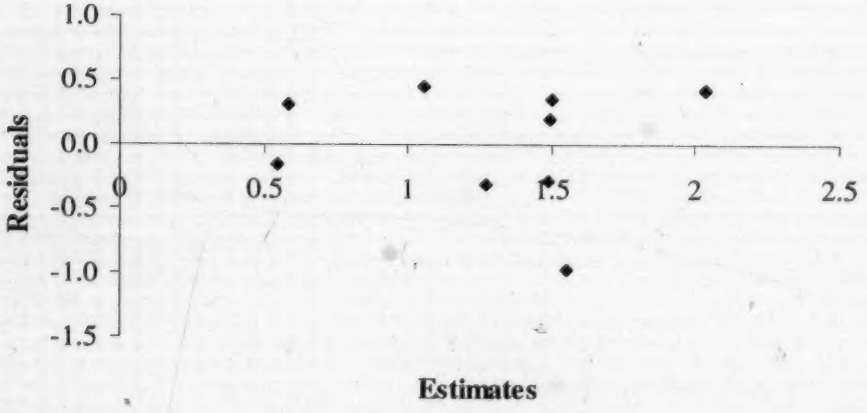

Figure 4-15 Residual – Estimate plot of Hom model

The reason that the Hom model fit the data better is the added exponent parameter in the time variable *t*. In general, a model with more parameters will tend to fit a dataset better than a model with fewer parameters. In this case however, the exponent on the *t* variable is particularly important because the experimental time values varied over a large range (i.e. 5 to 120 min) and had the greater influence on the $C_{avg}t$ values when compared to the influence of the ozone concentration (0.5 to 1.5 mg/L). The key to capturing the tailing effect at high $C_{avg}t$ values and achieving a good model fit, therefore, was the exponent on the time parameter. Note that the exponents on the C_{avg} and *t* parameters in the Hom model were almost the same. This suggests that inactivation curve could probably be adequately modeled with a single exponent on the concentration time product: i.e. $\log (N_0 /N) = k(C_{avg}t)^n$. This model is illustrated in Figure 4-16, below.

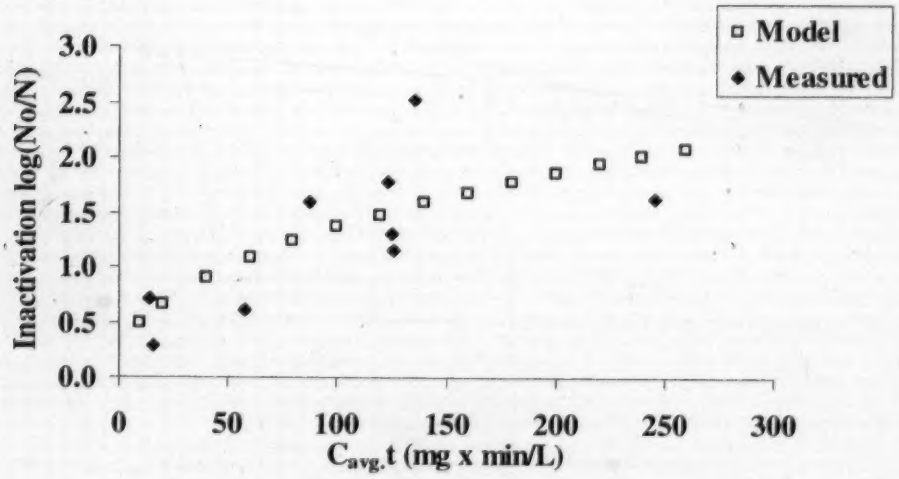

Figure 4-16 Comparison between measured biofilm HPC bacteria inactivation by ozone and that predicted using single exponent models

$$\text{Final model}: \log\frac{N_0}{N} = 0.174(C_{avg}\ t)^{0.447} \qquad \text{Equation 4-3}$$

Obviously, the final single exponent model also fit the data very well, as the analysis above confirmed. This relatively simple analysis suggests that the kinetic so inactivation of attached bacteria may be modeled using approaches that have been used to model inactivation of suspended bacteria by chemical disinfectants. Further, the models may be potentially be used to establish pipeline disinfection procedures and criteria.

4.4.3 Ozone disinfection efficacy of biofilm: a comparison between previous experimental data and this study data

Mathrani (2006) conducted a similar biofilm disinfection experiment previously. In his experiment, the biofilm ages ranged from 3 to 7 weeks, similar to the ages in this experiment, which were from 4 to 10 weeks. Moreover, both disinfection processes were performed in ODF phosphate buffer with pH 6.0. In addition, Mathrani performed the experiments using 16-20 week old biofilm with a pH of 6.9. Further, the biofilms used in the two experiments were all cultivated on the same material – cement coupons. However, the results of the two studies were quite different as the comparison in Figure 4-17 shows.

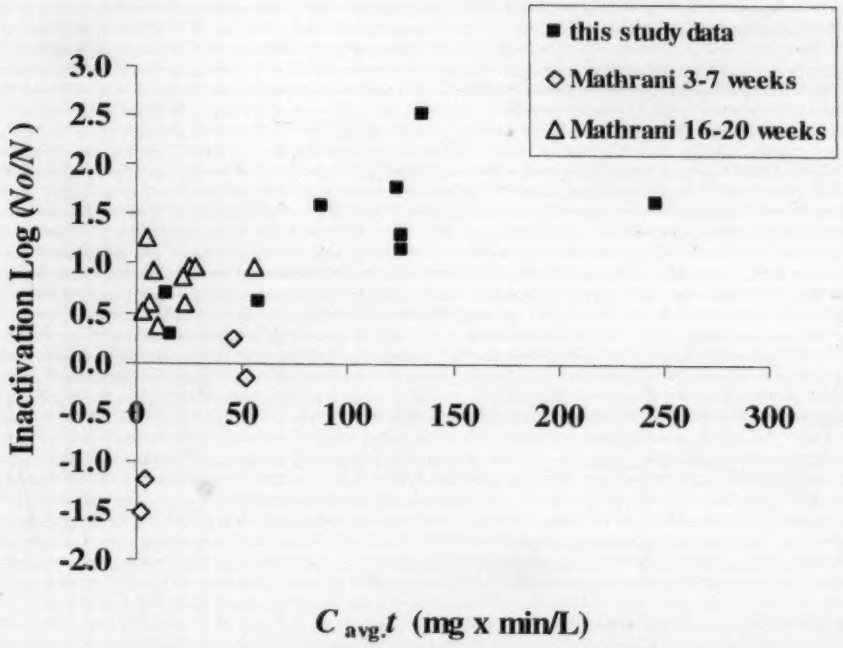

Figure 4-17 Comparison of biofilm HPC bacteria inactivation versus average ozone $C_{avg}t$ at 22°C measured in this study and Mathrani (2006) study

The same inactivation trends were observed in both experiments as show in figure 4-17. According to Mathrani's 3 to 7 weeks biofilm inactivation experiment, the inactivation was from -1.5 to 0.24 with C_{avg} t of 1.7 to 52.3 mg x min/ L. The negative value -1.5 means the measured number of surviving bacteria attached to the cement surface after disinfection was actually greater than the number of bacteria before disinfection.

According to Mathrani's 16 to 20 weeks biofilm disinfection experiment, the inactivation increased from 0.36 to 1.26 log with increasing $C_{avg}t$ from 3.2 to 56.3 mg x min /L. But his results contained substantial variability around the same $C_{avg}t$. For example, when $C_{avg}t$ was about 5 mg x min/L, the measured inactivation ranged from

0.51 to 1.26 log. In this study the measured results were less variable and the correlation with $C_{avg}t$ was stronger. Moreover, no unusual negative inactivation was observed. Many reasons could contribute to the differences. One of them might be the different biofilm removal and dispersion methods used. In Mathrani's experiment, the top layer of biofilm was removed from cement coupon into phosphate buffer using a sterilized robber policeman after exposure to the disinfectant. The solution was then homogenized prior to HPC enumeration. No surfactant dispersal solution was used. Mathrani suggested that the negative inactivation he observed for the 3 to 7 week old biofilms at low ozone $C_{avg}t$ was an artifact that was related to inefficient biofilm removal and dispersal techniques. Low ozone exposure may have simply reacted with the biofilm EPS, thereby weakening the biofilm structure without sufficient penetration of the biofilm to inactivate the bacteria. When a homogenization step was applied, the biofilm was readily dispersed and resulted in larger colony counts. In the present study, the coupons were scraped with a rubber policeman before the disinfectant experiment in order to remove loosely attached biofilm. After disinfection, the cement surface was brushed using a soft bristle brush and with the aid of Camper's dispersal solution. The solution was then homogenized and enumerated. This technique tended to produce much larger bacterial counts on the cement surface (10^6 CFU/cm^2 in this study versus 10^3 CFU/cm^2 in Mathrani's study) for biofilms that were cultured for similar lengths of time, even though loose biofilm was first removed in this study. This implies that the techniques used in this study were more effective at removal and dispersal of biofilm than those used by Mathrani (2006).

The pre-scraping step was adopted in this study in order to better simulate field conditions. During main break repair and rehabilitation, as practiced by Denver Water, a section of new main pipe is washed using a high pressure spray of water prior to installation and disinfection. During this procedure the surface layer of biofilm will likely by removed and washed out. The on-site disinfection is usually carried out after pipe flushing, and is intended to inactivate any biofilm or attached bacteria that remain on the pipe walls. The scraping step used in this study was intended to similarly remove the loosely attached biofilm, but probably did not remove bacteria that were more firmly attached or that were protected in surface cracks and crevices of the cement.

To imitate the real world conditions, the surface biofilm growth on the cement surfaces were removed prior to disinfection and were not enumerated in these experiments. Only the biofilm bacteria attached tightly to the coupon's surface were exposed to the disinfection process. The removal method was also modified. The biofilm remaining after disinfection was carefully removed by a sterilized toothbrush with the aid of a surfactant solution (Camper's solution). Using this protocol, the expected trend if increasing inactivation with increasing ozone $C_{avg.}t$, was observed. The new protocol developed in this thesis produced a more reliable and reproducible method of HPC biofilm removal, thus allowing a more accurate determination of bacterial inactivation in subsequent experiments.

The range of $C_{avg.}t$ studied were another significant difference between this study and Mathrani (2006). Mathrani's experiment did not investigate $C_{avg.}t$ values larger than 52.3 mg × min/L because he was using a semi-batch reactor for the ozone contact experiments with ozone solution feed but no ozone solution outlet. When the liquid level

reached the top of the reactor, the experiment had to be stopped. In this study, in contrast, the ozone reactor was designed as a continuous-flow reactor, with both inlet and outlet flow. The continuous-flow configuration combined with continuous ozone residual monitoring made it easy to control the ozone residual within the desired range and also allowed the ozone contact period to be as long as desired. An ozone $C_{avg}t$ of 250 mg x min/L was readily achieved. Compared to Mathrani's experiment, this experiment was more reliable and representative in terms of the imitation of actual field conditions and biofilm inactivation and provided more flexibility to study a larger range of $C_{avg}t$ values.

4.4.4 Comparison of ozone inactivation of biofilm bacteria and suspended bacteria

In order to further evaluate the effectiveness of ozone against biofilm bacteria, ozone inactivation of biofilm HPC bacteria determined in this study was compared to ozone inactivation data of suspended HPC bacteria available in the literature. Ozone inactivation data of suspended HPC bacteria was available from previous research reported by Gyürék and Finch (1998). The comparison is shown below:

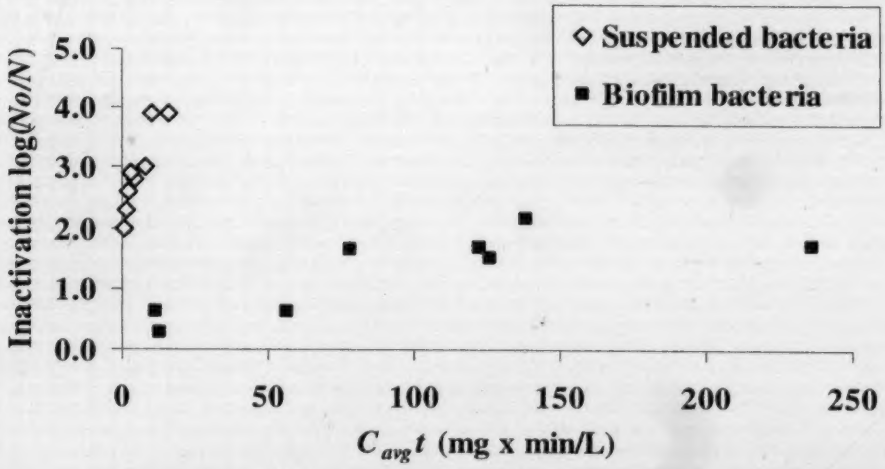

Figure 4-18 Comparison of ozone inactivation of suspended HPC bacteria (data from Gyürék and Finch, 1997) and biofilm HPC bacteria (data from this study)

Figure 4-18 shows that the ozone inactivation for suspended HPC bacteria was between 2 to 4 log in $C_{avg}t$ range of 0.46 to 16.11 mg x min/L at pH 6.9, as Gyürék and Finch (1997) reported. In comparison, the inactivation of biofilm HPC bacteria by ozone measured in this study was limited to about 2 log when the $C_{avg}t$ ranged from 11.33 to 236.4 mg x min/L at pH 6.0 conditions. At the same $C_{avg}t$ range, the inactivation efficiency of suspended HPC bacteria was at least 3 log greater than the inactivation of biofilm HPC bacteria (3.9 log for suspended bacteria versus 0.6 log for biofilm bacteria at $C_{avg}t$ around 10.0 mg x min/L, for example). Comparing the peak values, the same conclusion can be drawn. Inactivation of suspended bacteria at a $C_{avg}t$ of 10 mg x min/L, was 4 log while only 1.7 log of biofilm bacteria was measured at a $C_{avg}t$ was of 120 mg x min/L. In summary, ozone disinfection was considerably more effective for suspended HPC bacteria than for biofilm HPC bacteria. The possible reasons include:

(i) reaction-diffusion limitations may result in a much lower ozone concentration at the biofilm bacteria cell wall than in the bulk solution (Chen and Stewart, 1996);

(ii) biofilms appear to have a good intrinsic resistance system (multidrug efflux pump);

(iii) Extracellular enzymes of biofilm can physico-chemically quench biocide (Morton *et al.*, 1998).

LeChevallier *et al.* (1998) and Gyürék and Finch (1997) demonstrated that ozone is a powerful disinfectant for suspended bacteria. In LeChevallier *et al* study, four log inactivation can be reached at $C_{avg}t$ of 10 mg x min/L, while monochloramine needs a $C_{avg}t$ of 90 mg x min/L to reach 2 log inactivation rate at pH 7.0. However, with respect to biofilm bacteria, monochloramine was a powerful disinfectant due to its ability to penetrate the biofilm. Almost 3 log inactivation was achieved at 900 $C_{avg}t$. With ozone disinfection however, the highest inactivation observed in this study is 1.7 log at a $C_{avg}t$ of 120 mg x min/L. It may simply be that ozone is too reactive a molecule to penetrate to the biofilm to the extent required for complete inactivation.

4.4.5 Monochloramine Disinfection Experiments

The objective of these experiments was to expose biofilms grown on cement surfaces to a monochloramine solution with a target concentration of approximately 2.0 mg/L for periods of 6 and 24 hours. The target monochloramine concentration was 2.0 mg/L, because generally the residual of monochloramine in a distribution system is maintained at about 2.0 mg/L in order to provide residual protection against bacteria and

to prevent bacterial regrowth in distribution system water piping. Water utilities would find it very convenient if the potable water from another nearby existing water main can be taken advantage of to disinfect piping following repair or installation of new water mains.

Biofilm disinfection experiments with monochloramine were carried out in a 1000 mL well-mixed batch reactor flask. This solution was continuously stirred by using a magnetic stir bar to ensure well-mixed conditions. The concentration of monochloramine residual was measured at the beginning of the contact period, after 6 hours and after 24 hours using the DPD standard method. The actual monochloramine residual was between 2.0 to 2.5 mg/L (See Appendix A-12). The results of four disinfection experiments are shown in Figure 4-19.

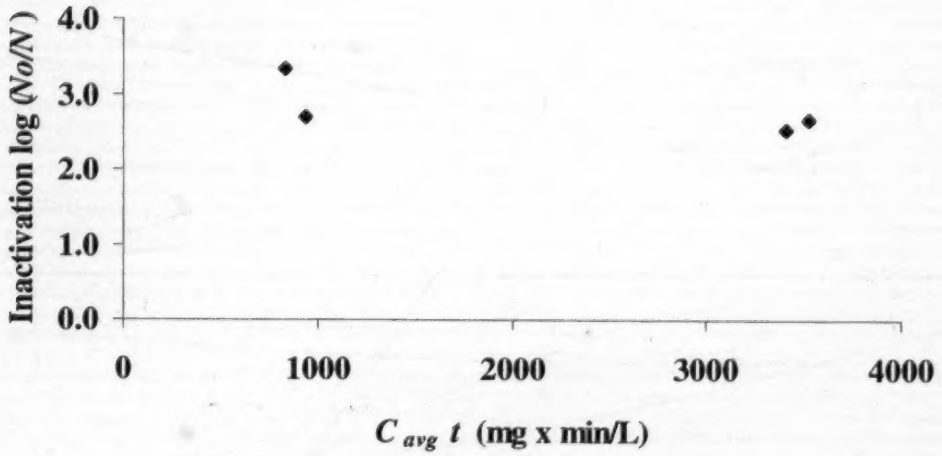

Figure 4-19 Inactivation of biofilm HPC bacteria versus monochloramine $C_{avg}t$ at 22°C, pH 7.5

As shown in Figure 4-20, about 2.5 to 3.5 log inactivation was observed after a $C_{avg}t$ of approximately 900 mg x min/L, but there was no evidence of an increase in log inactivation when the $C_{avg}t$ was increase to approximately 3,500 mg x min/L. This suggests that while many of the bacteria were readily inactivated by monochloramine, there were some highly resistant survivors. LeChevallier *et al.* (1990) conducted research on the effects of chlorine and monochloramine as disinfectants on biofilm bacteria on iron pipe from a drinking water distribution system. The average monochloramine residual was 2.0 mg/L. Their results indicated that two weeks contact time was necessary to inactivate significantly more than 3 log of bacteria in the biofilm. Momba *et al.* (2003) examined biofilm HPC bacteria grown on stainless steel and cement coupons. More than 2 log inactivation of microorganisms was achieved by 2.5 mg /L monochloramine inactivation for 24 hours. The results of this study are therefore, consistent with the results of previous studies.

LeChevallier *et al.* (1988) investigated both suspended and biofilm bacteria disinfection using chlorine and chloramine. In terms of unattached bacteria inactivation, the relative effectiveness of disinfectants tested was the following: $HOCl > ClO_2 > OCl^- > NH_2Cl$. When the $C_{avg.}t$ of monochloramine was equal to 94.0± 7.0 and the pH was 7.0, the disinfection efficiency could reach 99% (2 log). When $C_{avg.}t$ was 278.0 ± 46.0 and pH was 8.5, the inactivation was the same. The pH, therefore, has a significant effect on bacterial inactivation by monochloramine. In terms of attached bacteria inactivation, a comparison of equal activities of disinfectants for biofilms grown on metal coupons showed that monochloramine was more effective than the other disinfectants. A monochloramine $C_{avg.}t$ of 100 mg x min/L resulted in 99.5% (2 log) inactivation. At the

same $C_{avg}.t$ values, chlorine dioxide and hypochlorous acid could not reach 99.5% (2 log) inactivation. Comparing the results of that study with this study, a monochloramine $C_{avg}.t$ of approximately 900 mg x min/L resulted in 99.95% (3 log) HPC bacteria inactivation.

The results of the monochloramine biofilm disinfection experiments can also be compared with the results of the ozone biofilm disinfection results. Under the conditions tested, monochloramine resulted in 1 log more inactivation in comparison to ozone. The experimental data collected in this experiment was not sufficient to develop a mathematic model of monochloramine inactivation, however, it did suggest that an extended exposure to monochloramine may be a very effective strategy for disinfecting attached bacteria on pipe walls.

4.4.6 Disinfection effect comparison based on the viable bacteria species

The results of these disinfection experiments provided quantitative information about the inactivation of biofilm HPC bacteria treated by the different disinfectants. The bacterial plate counts provided qualitative information also. Based on color of the colonies observed on the plates, the types of surviving bacteria species where different when different disinfectants were used (Figure 4-20).

Figure 4-20 Photograph of HPC enumeration plates from ozone disinfection (left) and monochloramine disinfection (right) experiments

In Figure 4-20, the HPC plate on the left was from a 10^{-5} dilution after ozone disinfection. The presence of colonies of different colors including yellow, pink, orange, light blue and transparent indicates the present of different bacteria species in the population of survivors. The HPC plate on the right was from a 10^{-4} dilution after monochloramine disinfection. Only light blue colonies were observed on the plate containing bacteria exposed to monochloramine. The monochloramine treatment not only resulted in greater inactivation, but also seemed to inactivate a broader range of bacterial species. Williams *et al.* (2005) investigated the microbial diversity of distribution systems exposed to different disinfectants. They found that the biofilm communities in disinfection systems changed in response to disinfection practices. For example, biofilms exposed to chloramines mainly contained *Mycobacterium* species and

Dechloromonas species. While a variety of alpha- and additional beta-proteobacteria dominated in the biofilms exposed to free chlorine. Little is known about the diversity of microbial biofilm communities in new distribution system piping. The data provided from the results of this thesis suggest that monochloramine is able to inactivate a broader spectrum of bacterial species than ozone alone.

4.4.7 Sequential disinfection experiments

This research attempted to determine if the combination of the two disinfectants would provide a synergistic effect on biofilm HPC bacteria inactivation. As we know, ozone is a strong oxidant and reacts with the molecular components of the bacteria cell, but is relatively reactive and unstable. Monochloramine is a weaker disinfectant, but is more stable and has has good penetration ability. The hypothesis is that, when applied sequentially, ozone will first react with the EPS and will weaken the structure of the biofilm, making it more permeable. Monochloramine can then more readily penetrate deep into the biofilm layer and inactivate more bacteria. Biswas *et al.* (2005) reported that a synergistic effect was observed when ozone and monochloramine were applied sequentially to inactivate *Cryptosporidium parvum* in natural water.

Moreover, in practice Denver Water disinfects water mains using a trailer-mounted flow-through ozone system. After the ozone contact period, the ozone is flushed from the pipe using distribution system water that contains a monochloramine residual of about 2.0 mg/L in the distribution system. Sequential disinfection with ozone followed by a low concentration of monochloramine, therefore, is a realistic and practical treatment strategy. Based on this consideration and analysis, two sets of experiments

were conducted in which ozone contact was followed by a period of monochloramine contact. The results are shown in Tables 4-5 and 4-6. Biofilm coupons were exposed to solutions with approximately 1.0 mg/L ozone residual for 10 minutes (runs 1, 3, 5, and 6) and 120 minutes (runs 2, 4, and 7), respectively. The ozone exposure was followed by exposure to a 2.0 mg/L monochloramine solution for 24 hours.

Table 4-5 Results of 10 minute ozone inactivation followed by 24 hour monochloramine disinfection at 22°C.

Run No.	Ozone		Monochloramine		Total inactivation $\log(N_0/N)$
	pH	$C_{avg}t$ (mg x min/L)	pH	$C_{avg}t$ (mg x min/L)	
1	6.0	7.69	7.5	3744	4.84
3	6.0	13.36	7.5	3672	2.18
5	6.0	11.33	7.5	3355	4.61
6	6.0	12.57	7.5	3398	3.57

Table 4-6 Results of 120 minute ozone inactivation followed by 24 hour monochloramine disinfection at 22°C.

Run No.	Ozone		Monochloramine		Total inactivation $\log(N_0/N)$
	pH	$C_{avg}t$ (mg x min/L)	pH	$C_{avg}t$ (mg x min/L)	
2	6.0	117	7.5	3528	3.26
4	6.0	138	7.5	3413	3.15
7	6.0	124	7.5	3398	0.51

An unusually low inactivation result of 0.51 log was obtained in Run 7. The previous experiments had indicated that single disinfectant contact with 120 mg × min/L

ozone contact or 3,600 mg × min/L monochloramine should achieve more than 1.5 log inactivation. Therefore, this result was considered suspect. In this suspect trial, the HPC enumeration plate contained colonies of more than one colour, while in all other HPC plates after sequential ozone and monochloramine treatment, the plates contained colonies of only one colour. Therefore, contamination was suspected for this suspect trial (see Figure 4-21). Tables 4-5 and 4-6 indicated that an average 3.8 log bacteria reduction was obtained in the first disinfectant combination, and 3.2 log bacteria inactivation was achieved by the second disinfectant combination (if the suspect result of Run 7 is omitted). It seems that in the sequential disinfection process, the microorganism inactivation decreased when the ozone primary treatment $C_{avg}.t$ values increased. However, given the variability in the data and the limited number of experiments conducted, it is not possible to conclude that there was a statistical difference between the ozone primary treatment levels.

Table 4-7 shows the predicted biofilm HPC bacteria inactivation from sequential exposure to monochloramine based on a calculation that assumes and additive effect of ozone and monochloramine exposure. The ozone inactivation was calculated using the Hom model generated for ozone disinfection of biofilm HPC bacteria (Equation 4-3). When average experimental ozone conditions was $C_{avg}.t$ of 11.2 and 126 mg x min/ L, the calculated inactivation was equal to 0.51 and 1.51 log, respectively. The additional monochloramine inactivation was assumed to be 2.5 based on the monochloramine experimental results of Figure 4-20. The calculated inactivation based on an additive effect is compared to the mean inactivation measure in the experiments (Tables 4-6 and 4-7).

Table 4-7 Comparison of observed biofilm inactivation after sequential disinfection with ozone and monochloramine and inactivation calculated based on an additive effect.

Ozone $C_{avg}t$ (mg x min/L)	Monochloramine $C_{avg}t$ (mg x min/L)	Inactivation Log(N_0/N) (calculated)	Inactivation Log(N_0/N) (observed)
11.2	3542	3.01	3.80
126	3446	4.01	3.33

As table 4-7 shows, the calculated total additive inactivation of biofilm bacteria based after exposure to ozone for 10 min followed by exposure to monochloramine for 24 is slightly greater than 3 log. At the lower ozone exposure, the average measured inactivation for sequential treatment was 3.8 log, which suggests a synergistic effect. At the higher ozone exposure, the average measured biofilm HPC bacteria inactivation after 120 minutes ozone exposure followed by 24 hours monochloramine exposure was almost 1 log less than the inactivation calculated based on an additive effect. Therefore, there may have been a synergistic effect at the lower ozone pre-treatment level but there was no evidence of a synergistic effect at the higher ozone pre-treatment level. Overall, given the variability in the outcomes, there was not enough data to conclude that a synergistic effect was present at either ozone treatment level.

These experiments to determine if there was a synergistic effect or not when multiple disinfectants are used to inactivate biofilm in distribution system, therefore, were inconclusive. Additional experiments using different sequential disinfectant combinations should be conducted in future research.

5 Conclusions and Recommendations

5.1 Conclusions

In this study, the effectiveness of ozone and monochloramine for inactivating biofilm HPC bacteria grown on the cement-mortar coated plastic coupons was studied. The cement-mortar surfaces were used to simulate the interior walls of cement-mortar line pipe used for distribution system rehabilitation and repairs. The following conclusions were drawn from this project:

- Maximal recovery of bacteria from biofilms grown on cement-mortar coupons was achieved by a combination of physical scraping with a sterilized brush into a surfactant solution (Camper's solution). The bacteria were then dispersed with a homogenizer for 2 mins set at 25,000 rpm. This combination resulted in the greatest number of enumerated HPC bacteria.
- When biofilms were treated with ozone, the relationship between log inactivation and aqueous ozone $C_{avg}t$ in the bulk liquid was not linear. A non-linear Hom model was used to fit the data. The highest inactivation achieved was 1.7 log at and ozone $C_{avg}t$ of 120 mg x min/L. At higher $C_{avg}t$ values, tailing was observed in the inactivation curve. Increasing the disinfectant exposure to 240 $C_{avg}t$ did not increase disinfection efficiency. Inactivation of biofilm HPC bacteria using ozone, therefore, is expected to be limited to less than 2 log.
- When biofilms were treated with low concentration (about 2 to 2.5 mg/L) monochloramine, 2.7 log inactivation was achieved after 6 h and 24 h of exposure. An extended (6 to 24 h) exposure to a concentration of

monochloramine typical of distribution system water (2 to 2.5 mg/L), therefore, is more effective than a shorter (120 min) exposure to ozone (1.0 to 1.5 mg/L) for disinfection of biofilms on cement surfaces. The relative chemical stability and penetrating ability may provide monochloramine with certain advantages as a pipeline disinfectant material.

- There was evidence of a synergistic inactivation effect when biofilm HPC bacteria grown on cement surfaces were exposed to ozone followed by monochloramine but the result was statistically inconclusive. This may have been due to the rather limited dataset.

5.2 Recommendations

According to the experimental procedures and results, the following recommendations need to be considered in future investigations:

- Only one ozone disinfection experiment was carried out at the largest $C_{avg}t$ of 240 mg x min/L due to limited availability of experimental biofilm samples. Additional experiments should be conducted at an ozone $C_{avg}t$ of 240 mg x min/L to better define the inactivation $C_{avg}t$ relationship at higher $C_{avg}t$ values.
- The disinfection experiments in these studies were carried out using HPC bacteria grown on only two pipe wash water samples collected from the Denver Water pipe yard. More pipe wash water samples should be taken from different pipe yards and different seasons in order to determine to what extent the disinfection results will apply to a broader mix of HPC bacterial populations that might be encountered in the field.

- Additional experiments should be done on inactivation of biofilm HPC bacteria with monochloramine. These additional experiments should concentrate at lower $C_{avg}.t$ values in order to determine the relationship between $C_{avg}.t$ and inactivation at shorter exposure times and to develop a kinetic model. At each $C_{avg}.t$ value, more replicate experiments are required in order to draw statistical conclusions.
- Identifying bacterial species of biofilm grown in actual distribution systems would be very helpful to choose appropriate disinfectants for pipeline disinfection.
- To further investigate a synergistic effect, and to determine if the effect is statistically significant, additional experiments should be conducted using different combinations of ozone followed by monochloramine.

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Appendix A Raw Experimental Data

Table A-1 Measured properties of the pipe wash water sample periodically supplied by Denver Water

Sample Date	Replicate	pH	Free Cl_2 (mg Cl_2/L)	Turb. (NTU)	COD mg O_2/L		HPC CFU/100ml
					Abs.	Conc.	
Oct.12/05	1	6.15	0.001	45.6	0.124	792.63	6.3×10^8
	2	6.10	0.000	45.3	0.123	786.24	6.2×10^8
	3	—	0.001	—	0.118	754.28	6.1×10^8
	Average	6.125	0.001	45.5	0.122	777.72	6.2×10^8
Feb.14/06	1	6.81	0.084	5.39	0.033	94.58	6.6×10^8
	2	6.62	0.080	5.55	0.027	77.38	6.7×10^8
	3	6.72	0.084	5.12	0.022	63.05	6.9×10^8
	Average	6.72	0.082	5.33	0.027	77.38	6.8×10^8
Mar.24/06	1	6.18	0.191	42.4	0.14	2011.38	N/A
	2	6.24	0.183	40.9	0.136	1953.91	N/A
	3	6.22	N/A	42.6	0.139	1997.01	N/A
	Average	6.21	0.187	41.97	0.138	1987.44	N/A

Note: N/A means not available.

Table A-2 Conditions of biofilm growth (the first biofilm growth experiment)

Date	Rep.	pH	DO (mg O_2/L)	Turb. (NTU)	Temp. °C	COD mg O_2/L	Note
Oct.12/05	1	6.15	—	45.6	—	—	
	2	6.10	—	45.3			
	3	—	—	—	—	—	
	Avg.	6.13	—	—	—	—	
Oct.13/05	1	6.22	6.81	4.6	22.9	300.43	P=29.8inch Cs=7.87mg O_2/L
	2	6.20	6.86	4.3	22.9	290.85	
	3	6.20	—	4.6	22.9	294.04	
	Avg.	6.21	6.84	4.5	22.9	295.11	
Oct.14/05	1	—	—	—	—	332.39	
	2	—	—	—	—	319.61	
	3	—	—	—	—	332.39	
	Avg.	—	—	—	—	328.13	
Oct.17/05	1	6.16	9.03	2.48	22.2	396.32	P=29.7inch Cs= 7.83 mgO_2/L
	2	6.18	8.96	2.54	22.2	383.53	
	3	6.16	—	2.52	22.2	386.73	
	Avg.	6.17	9.00	2.51	22.2	388.86	
Oct.18/05	1	—	—	—	—	380.34	
	2	—	—	—	—	377.14	
	3	—	—	—	—	437.87	
	Avg.	—	—	—	—	398.45	
Oct.19/05	1	—	—	—	—	377.14	
	2	—	—	—	—	380.34	
	3	—	—	—	—	377.14	
	Avg.	—	—	—	—	378.21	
Oct.20/05	1	6.22	7.40	2.12	22.2	396.32	P=30.1inch Cs= 7.80mgO_2/L
	2	6.23	7.48	2.10	22.2	396.32	
	3	6.24	7.48	2.14	22.2	377.14	
	Avg.	6.23	7.45	2.12	22.2	389.92	
Oct 24/05	1	6.27	7.79	2.29	22.7	389.92	P=30.0inch Cs= 7.77mgO_2/L
	2	6.26	7.82	2.12	22.7	370.75	
	3	6.25	—	2.10	22.7	373.94	
	Avg.	6.26	7.81	2.17	22.7	378.21	
Oct.25/05	1	—	—	—	—	351.57	
	2	—	—	—	—	389.92	
	3	—	—	—	—	348.37	
	Avg.	—	—	—	—	363.29	

Table A-2 (Continued)

Date	Rep.	pH	DO (mg O2/L)	Turb. (NTU)	Temp. oC	COD (mg O2/L)	Note
Oct.27/05	1	6.31	7.5	3.09	22.4	345.18	P=30.0inch Cs= 7.77mgO2/L,
	2	6.29	7.51	3.04	22.4	364.36	
	3	6.30	—	3.08	22.4	386.73	
	Avg.	6.30	—	3.07	22.4	365.42	
Oct.31/05	1	6.33	7.8	3.5	22.3	378.51	P=29.8inch Cs= 7.78mgO2/L T=23.6 oC,
	2	6.33	7.9	3.83	22.3	313.45	
	3	6.32	—	3.68	22.3	283.88	
	Avg.	6.33	7.85	3.67	22.3	325.28	
Nov.02/05	1	6.3	7.43	3.54	22.6	266.14	P=29.8inch Cs= 7.78mgO2/L T=23.6 oC,
	2	6.29	7.42	3.68	22.6	263.18	
	3	6.28	—	3.6	22.6	257.27	
	Avg.	6.29	7.425	3.61	22.6	262.20	
Nov.07/05	1	6.31	8.7	3.54	22	233.61	P=30.0inch Cs= 7.86mgO2/L T=23.4oC
	2	6.28	8.79	3.49	22	239.53	
	3	6.31	—	3.64	22	224.74	
	Avg.	6.30	8.745	3.56	22	232.63	
Nov.10/05	1	6.33	7.89	8.53	22.1	260.22	P=29.4inch Cs= 7.60mgO2/L T=23.9oC
	2	6.31	7.97	8.71	22.1	269.10	
	3	6.33	—	8.70	22.1	254.31	
	Avg.	6.32	7.93	8.6467	22.1	261.21	
Nov.14/05	1	6.42	8.01	9.72	22.1	245.44	P=30.4inch Cs= 7.96mgO2/L T=23.5oC
	2	6.42	8.02	9.48	22.1	242.48	
	3	6.39	—	9.47	22.1	245.44	
	Avg.	6.41	8.02	9.56	22.1	244.45	
Nov.17/05	1	6.44	7.09	13.09	22.2	245.44	P=30.1inch Cs= 7.93mgO2/L T=23.2oC
	2	6.41	7.11	13.16	22.2	257.27	
	3	6.42	—	13.01	22.2	242.48	
	Avg.	6.42	7.10	13.09	22.2	248.40	
Nov.21/05	1	6.44	6.68	11.61	22.1	227.70	P=30.1inch Cs= 8.05mgO2/L T=22.4oC
	2	6.43	6.71	12.21	22.1	221.78	
	3	6.43	—	11.83	22.1	221.78	
	Avg.	6.43	6.70	11.88	22.1	223.75	
Nov.24/05	1	6.49	7.67	8.72	21.4	198.13	P=29.7inch Cs=7.90mgO2/L T=22.7oC
	2	6.48	7.60	8.48	21.4	201.08	
	3	6.49	—	8.64	21.4	189.25	
	Avg.	6.49	7.64	8.61	21.4	196.15	

Table A-2 (Continued)

Date	Rep.	pH	DO (mg O_2/L)	Turb. (NTU)	Temp. °C	COD mg O_2/L	Note
Nov.28/05	1	6.58	8.22	6.64	21.5	251.35	P=30.5inch Cs=8.03mgO_2/L, T=23.2°C
	2	6.56	8.31	6.64	21.5	254.31	
	3	6.56	—	6.69	21.5	257.27	
	Avg.	6.57	8.25	6.66	21.5	254.31	
Dec.05/05	1	6.84	9.28	14.51	22.1	147.86	P=30.5inch Cs=7.80mgO_2/L T=24.3°C
	2	6.81	9.29	14.45	22.1	165.60	
	3	6.81	—	14.38	22.1	159.68	
	Avg.	6.82	9.28	14.45	22.1	157.71	
Dec.08/05	1	6.9	7.97	29.1	21.2	198.13	P=30.0inch Cs=7.96mgO_2/L T=22.7°C
	2	6.9	7.99	29.4	21.2	204.04	
	3	7.04	—	29.6	21.2	189.25	
	Avg.	7.00	7.98	29.40	21.2	197.14	
Dec.12/05	1	7.01	8.96	18.4	22.1	171.51	P=29.8inch Cs=7.70mgO_2/L T=24.3°C
	2	7.01	8.92	16.4	22.1	168.55	
	3	6.99	—	18.6	22.1	171.51	
	Avg.	7.00	8.94	17.80	22.1	170.53	
Dec.22/05	1	7.1	8.54	9.86	22.1	115.33	P=29.4inch Cs=7.59mgO_2/L T=24.1°C
	2	7.09	8.55	9.43	22.1	118.28	
	3	7.08	—	8.05	22.1	115.33	
	Avg.	7.09	8.55	9.11	22.1	116.31	
Jan.03/06	1	7.28	8.43	7.06	21.3	109.41	P=29.8inch Cs=7.83mgO_2/L T=23.2°C
	2	7.31	8.45	6.81	21.3	112.37	
	3	7.3	—	6.93	21.3	106.46	
	Avg.	7.3	8.44	6.93	21.3	109.41	
Jan.10/06	1	7.52	8.0	9.62	22.4	85.76	P=29.9inch Cs=7.78mgO_2/L T=23.7°C
	2	7.51	7.98	9.4	22.4	91.67	
	3	7.5	—	9.52	22.4	91.67	
	Avg.	7.51	7.99	9.51	22.4	89.70	
Jan.23/06	1	7.69	7.51	8.13	22.1	100.54	P=29.6inch Cs=7.76mgO_2/L, T=23.6°C
	2	7.69	7.52	8.01	22.1	91.67	
	3	7.69	—	7.71	22.1	91.67	
	Avg.	7.69	7.52	7.95	22.1	94.63	

Table A-3 Conditions of biofilm growth (the second biofilm growth experiment)

Date	Rep.	pH	DO (mg O_2/L)	Turb. (NTU)	Temp. °C	COD mg O_2/L		Note
						Abs. at 600	Conc.	
Feb 17/06	1	6.81	—	5.39	—	0.032	91.95	Original water sample properties
	2	6.62	—	5.55	—	0.027	77.58	
	3	6.72	—	5.12	—	0.022	63.21	
	Avg.	6.72	—	5.35	—	0.026	74.71	
Feb.23/06	1	6.88	11.21	3.83	22.1	0.034	97.70	P=30.1inch T=23.9°C Cs=7.81mgO_2/L
	2	6.87	10.93	3.92	22.1	0.037	106.32	
	3	6.85	—	3.71	22.1	0.032	91.95	
	Avg.	6.87	11.07	3.82	22.1	0.0343	98.65	
Feb.27/06	1	7.16	8.06	2.93	21.2	0.036	103.44	P=29.6inch T=22.4°C Cs=7.89mgO_2/L
	2	7.14	7.93	2.84	21.2	0.034	97.70	
	3	7.13	—	2.87	21.2	N/A	N/A	
	Avg.	7.14	8	2.86	21.2	0.035	100.00	
Mar02/06	1	7.19	8.26	3.04	21.5	0.033	94.82	P=30.5inch T= 23.4°C Cs=8.00mgO_2/L.
	2	7.21	8.14	3.09	21.5	0.035	100.57	
	3	7.17	—	3.02	21.5	0.034	97.70	
	Avg.	7.19	8.2	3.05	21.5	0.034	97.70	
Mar06/06	1	7.29	7.04	3.3	21.9	0.023	66.088	P=29.9inch T= 23.3°C Cs=7.84mgO_2/L.
	2	7.28	7.13	3.5	21.9	0.021	60.341	
	3	7.28	—	3.45	21.9	0.022	63.215	
	Avg.	7.28	7.18		21.9	0.022	63.215	
Mar09/06	1	7.35	6.74	2.26	20.7	0.016	45.974	P=29.1inch T= 21.6°C Cs=8.07mgO_2/L.
	2	7.32	6.56	2.2	20.7	0.018	51.721	
	3	7.32	—	2.13	20.7	0.017	48.848	
	Avg.	7.33	6.65	2.20	20.7	0.017	48.848	
Mar13/06	1	7.44	6.71	1.41	20.6	0.011	31.607	P=20.1inch T= 20.6°C Cs=8.10mgO_2/L.
	2	7.42	6.72	1.37	20.6	0.01	28.734	
	3	7.42	—	1.4	20.6	0.009	25.861	
	Avg.	7.43	6.72	1.39	20.6	0.01	28.734	
Mar16/06	1	7.42	7.06	1.55	21	0.009	25.861	P=30.2inch T= 22.0°C Cs=8.13mgO_2/L.
	2	7.42	6.18	1.59	21	0.009	25.861	
	3	7.4	—	1.56	21	0.009	25.861	
	Avg	7.41	6.62		21	0.009	25.861	

Table A-3 (Continued)

Date	Rep.	pH	DO (mg O_2/L)	Turb. (NTU)	Temp. °C	COD mg O_2/L		Note
						Abs. at 600	Conc.	
Mar 20/06	1	7.58	7.1	1.16	20.6	0.011	31.607	P=30.2inch T= 21.7°C Cs=8.18mgO_2/L.
	2	7.56	6.96	1.14	20.6	0.006	17.24	
	3	7.58	—	1.14	20.6	0.01	28.734	
	Avg.	7.5	6.98	1.15	20.6	0.009	25.861	
Mar 23/06	1	7.7	7.17	0.62	19.9	0.009	25.861	P=30.4inch T= 21.9oC Cs=8.21mgO_2/L.
	2	7.65	7.14	0.63	19.9	0.007	20.114	
	3	7.64	—	0.62	19.9	0.006	17.24	
	Avg.	7.66	7.15	—	19.9	0.007	20.114	
Mar 27/06	1	7.69	7.16	0.61	20.2	0.009	25.861	P=30.0inch T= 20.6°C Cs=8.30mgO_2/L.
	2	7.78	6.53	0.56	20.2	0.006	17.24	
	3	7.7	—	0.55	20.2	0.007	20.114	
	Avg.	7.73	76.85	0.68	20.2	0.007	20.114	
Mar 30/06	1	7.8	6.3	0.64	20.8	0.008	22.989	P=29.9inch T= 21.7°C Cs=8.09mgO_2/L.
	2	7.86	5.71	0.66	20.8	0.014	40.23	
	3	7.8	—	0.66	20.8	0.008	22.989	
	Avg.	7.82	6	0.66	20.8	0.01	28.736	
Apr.03/06	1	7.94	7.38	0.52	20.6	0.009	25.862	P=30.0inch T= 21.1°C Cs=8.21mgO_2/L.
	2	7.94	6.81	0.51	20.6	0.007	20.115	
	3	7.93	—	0.49	20.6	0.007	20.115	
	Avg.	7.94	7.05	0.51	20.6	0.008	22.989	
Apr.06/06	1	7.89	9.23	0.68	19.9	0.011	31.607	P=29.9inch T= 20.5°C, Cs=8.28mgO_2/L.
	2	7.88	8.51	0.67	19.9	0.015	43.101	
	3	7.83	—	0.67	19.9	0.02	57.468	
	Avg.	7.86	8.86	0.67	19.9	0.0153	44.059	
Apr.10/06	1	7.71	8.71	0.72	19.9	0.023	66.088	P=29.7inch T= 21.8°C, Cs=8.01mgO_2/L.
	2	7.65	7.52	0.68	19.9	0.022	63.215	
	3	7.64	—	0.7	19.9	0.022	63.215	
	Avg.	7.67	—	0.7	19.9	0.022	63.215	
Apr.13/06	1	7.59	11.29	0.68	21	0.011	31.61	P=29.6inch T= 20.3°C, Cs=8.22mgO_2/L.
	2	7.64	10.69	0.68	21	0.01	28.736	
	3	7.65	—	0.71	21	0.008	22.989	
	Avg.	7.63	—	0.69	21	0.009	25.862	

Table A-3 (Continued)

Date	Rep.	pH	DO (mg O_2/L)	Turb. (NTU)	Temp. °C	COD mg O_2/L		Note
						Abs. at 600	Conc.	
Apr.17/06	1	7.5	8.4	0.6	20.9	0.022	63.219	P=29.9inch T= 20.3°C, Cs=8.03mgO_2/L
	2	7.53	8.33	0.6	20.9	0.012	34.483	
	3	7.54	—	0.53	20.9	0.011	31.61	
	Avg.	7.52	8.36	—	20.9	0.012	34.483	
Apr.20/06	1	7.74	8.66	0.6	20.3	0.118*		P=30.3inch T= 22.2°C, Cs=8.06mgO_2/L
	2	7.73	8.46	0.69	20.3	0.029	83.334	
	3	7.73	—	0.61	20.3	0.028	80.461	
	Avg.	7.73	8.56	0.63	20.3	0.029	81.79	
Apr.24/06	1	7.71	5.25	0.58	20.6	0.023	66.088	P=29.9inch T= 22.4°C, Cs=8.13mgO_2/L
	2	7.71	5.02	0.68	20.6	0.017	48.848	
	3	7.72	—	0.62	20.6	0.021	60.341	
	Avg.	7.71	5.13	0.63	20.6	0.020	58.426	
Apr.27/06	1	7.71	8.29	0.58	21.1	0.035	100.57	P=29.9inch T= 22.4°C, Cs=87.9mgO_2/L
	2	7.69	8.48	0.63	21.1	0.026	74.708	
	3	7.68	—	0.57	21.1	0.029	83.329	
	Avg.	7.69	8.38		21.1	0.03	86.202	
May01/06	1	7.81	7.77	0.6	21.1	0.035	100.57	P=29.9inch T= 22.4°C, Cs=7.98mgO_2/L
	2	7.78	7.59	0.61	21.1	0.026	74.708	
	3	7.78	—	0.64	21.1	0.027	77.582	
	Avg.	7.79	7.63	0.62	21.1	0.029	84.286	
May04/06	1	7.79	7.3	0.71	20.9	0.004*	—	P=30.1inch T= 21.9°C, Cs=8.12mgO_2/L
	2	7.78	11.73	0.68	20.9	0.016	45.97	
	3	7.77	—	0.66	20.9	0.006*	—	
	Avg.	7.78	—	0.68	20.9	0.016	45.974	
May07/06	1	7.83	6.78	0.61	20	0.019	54.595	P=29.7inch T= 20.8°C, Cs=8.17mgO_2/L
	2	7.83	7.19	0.63	20	0.029	83.329	
	3	7.84	—	0.7	20	0.028	80.455	
	Avg.	7.83	6.99	0.64	20	0.025	72.793	
May.11/06	1	7.75	9.38	0.59	21	0.014	40.228	P=30.1inch T= 22.4°C, Cs=8.04mgO_2/L
	2	7.78	9.06	0.55	21	0.015	43.101	
	3	7.79	—	0.63	21	0.01	28.734	
	Avg.	7.77	9.22	0.59	21	0.013	37.354	

Note: * means contaminated or unreasonable value

Table A-3 (Continued)

Date	Rep.	pH	DO (mg O_2/L)	Turb. (NTU)	Temp. °C	COD mg O_2/L		Note
						Abs. at 600	Conc.	
May16/06	1	7.79	6.37	0.63	21.1	0.012	34.481	P=30.2inch T= 22.0°C, Cs=8.13mgO_2/L.
	2	7.81	6.12	0.59	21.1	0.016	45.974	
	3	7.81	—	0.61	21.1	0.015	43.101	
	Avg.	7.8	6.25	0.61	21.1	0.014	41.185	
May19/06	1	7.83	6.9	0.71	21.3	0.034	97.696	P=29.9inch T= 22.2°C, Cs=8.01mgO_2/L.
	2	7.81	6.76	0.71	21.3	0.036	103.44	
	3	7.81	—	0.75	21.3	0.04	114.94	
	Avg.	7.82	6.83	0.72	21.3	0.037	105.36	
May23/06	1	7.74	7.82	0.76	21.2	0.048	137.92	P=29.7inch T= 21.7°C, Cs=8.01mgO_2/L.
	2	7.76	7.66	0.78	21.2	0.045	129.3	
	3	7.78	—	0.78	21.2	0.043	123.56	
	Avg.	7.76	7.74	0.77	21.2	0.045	129.3	
May25/06	1	7.71	7.55	0.72	20.0	0.054	155.16	P=29.9inch T= 21.5°C, Cs=8.12mgO_2/L.
	2	7.75	7.28	0.73	20.0	0.037	106.32	
	3	7.76	—	0.72	20.0	0.043	123.56	
	Avg.	7.74	7.42	0.72	20.0	0.045	128.35	
May29/06	1	7.85	7.92	0.83	20.0	0.034	97.696	P=29.9inch T= 22.1°C, Cs=8.03mgO_2/L.
	2	7.89	7.58	0.83	20.0	0.036	103.44	
	3	7.9	—	0.83	20.0	0.035	100.57	
	Avg.	7.88	7.75	0.83	20.0	0.035	100.57	
Jun.01/06	1	7.82	8.02	0.82	20.0	0.028	80.455	P=29.9inch T= 21.4°C, Cs=8.14mgO_2/L.
	2	7.87	7.72	0.79	20.0	0.025	71.835	
	3	7.88	—	0.79	20.0	0.035	100.57	
	Avg.	7.86	7.87	0.80	20.0	0.029	84.286	
Jun.05/06	1	7.84	7.8	1.1	20.2	0.038	109.19	P=29.8inch T= 22.1°C, Cs=8.00mgO_2/L.
	2	7.86	7.74	1.01	20.2	0.037	106.32	
	3	7.89	—	1.04	20.2	0.04	114.94	
	Avg.	7.86	7.77	1.05	20.2	0.038	110.15	
Jun.08/06	1	7.78	9.29	1.09	20.2	0.039	112.06	P=30.1inch T= 22.1°C, Cs=8.07mgO_2/L.
	2	7.85	9.35	1.11	20.2	0.041	117.81	
	3	7.85	—	1.05	20.2	0.041	117.81	
	Avg.	7.83	9.32	1.08	20.2	0.040	114.94	
Jun.08/06	1	7.93	9.19	1.19	19.7	0.032	91.949	P=30.2inch T= 21.4°C, Cs= 8.23mgO_2/L.
	2	7.89	8.48	1.19	19.7	0.037	106.32	
	3	7.9	—	1.18	19.7	0.037	106.32	
	Avg.	7.91	8.9	1.19	19.7	0.035	101.53	

Table A-4 Data from biofilm homogenization and dispersion experiments (CFU/100mL)

Replicate	Dispersion reagent	0.05M, ph=6.0, Phosphate Buffer											
	Homogenizer Speed	13500 rpm											
	Time (min)	2				6				10			
	Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
1	$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-5)}$	TNTC	TNTC	TNTC	TNTC	155	230	TNTC		118	270	*	
	$10^{(-6)}$	40	51	47	46	28	33	280	30	47	27	25	33
2	Time (min)	2				6				10			
	Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
	$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-5)}$	170	200	140	170	230	170	240	213.3	200	190	230	206.7
	$10^{(-6)}$	37	31	33	33								

Replicate	Dispersion reagent	0.01M, ph=7.0, Tris Buffer + 0.01% peptone + 10-6M Zwittergent											
	Homogenizer Speed	25000 rpm											
	Time (min)	2				6				10			
	Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
1	$10^{(-3)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-5)}$	123	90	47		89	TNTC	TNTC	89	TNTC	TNTC	TNTC	TNTC
	$10^{(-6)}$	20	*	160		188	TNTC	TNTC	TNTC	125	101	TNTC	TNTC
2	Time (min)	2				6				10			
	Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
	$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-5)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-6)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

Table A-4 (Continued)

Replicate	Dispersion reagent	0.01M, ph=7.0, Tris Buffer + 0.01% peptone + 10-6M Zwittergent (Camper's Solution)								Homogenizer Speed		13500 rpm	
1	Time (min)	2				6				10			
	Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
	$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-5)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-6)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
2	Time (min)	2				6				10			
	Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
	$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-5)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-6)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

Replicate	Dispersion reagent	0.05M, ph=6.0, Phosphate Buffer								Homogenizer Speed		25000 rpm	
1	Time (min)	2				6				10			
	Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
	$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-5)}$	290	160	170	210	80	130	70	90	100	50	270	140
	$10^{(-6)}$	28	23	43	32	46	22	26	31	39	26	29	31
2	Time (min)	2				6				10			
	Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
	$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	$10^{(-5)}$	230	220	108	186	220	220	200	213	220	230	230	227
	$10^{(-6)}$	29	42	40	37	39	28	24	30	49	19	31	33

Table A – 5 Regression model of data from biofilm dispersion experiments using phosphate buffer only

10^{-6} HPC CFU/100ml	Speed (rpm)	Time(min)	
Y	X1	X2	X1X2
42	13500	2	27000
52	25000	6	150000
33	13500	10	135000
48	25000	10	250000
31	13500	6	81000
48	25000	2	50000

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.9465633
R Square	0.8959821
Adjusted R Square	0.7399554
Standard Error	4.4064347
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	3	334.5	111.5	5.742489	0.151896157
Residual	2	38.83333333	19.417		
Total	5	373.3333333			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	33.572464	13.14685658	2.5536	0.125192	-22.99389459	90.138822
X_1	0.0006304	0.000654384	0.9634	0.436996	-0.002185154	0.003446
X_2	-2.4456522	1.924502745	-1.2708	0.331615	-10.72611916	5.8348148
X_1X_2	9.783E-05	9.57921E-05	1.0212	0.414563	-0.000314334	0.00051

Table A-6 Raw data from ozone disinfection experiments of biofilm HPC bacteria in 0.05M ODF phosphate buffer at 22°C.

Replicate	1								2							
Treatment	Before disinfection N_0				After disinfection N				Before disinfection N_0				After disinfection N			
Time (min)	15				15				15				15			
Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
$10^{(-2)}$					TNTC	TNTC	TNTC	TNTC					TNTC	TNTC	TNTC	TNTC
$10^{(-3)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
$10^{(-5)}$	94	97	96	96	58	53	40	51	153	159	178	163	53	48	56	52
$10^{(-6)}$	7	10	10	9					36	28	23	29				
Time (min)	60				60				33				33			
Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
$10^{(-2)}$													TNTC	TNTC	TNTC	TNTC
$10^{(-3)}$					TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	245	340	245	277
$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	42	56	47	48
$10^{(-5)}$	TNTC	TNTC	TNTC	TNTC	88	115	97	100	136	200	190	175	17	9	5	10
$10^{(-6)}$	57	37	42	45	11	9	56		37	19	24	27				
$10^{(-7)}$	6	8	8	7					5	8	6	6				
Time (min)	120				120				110				110			
Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
$10^{(-2)}$					TNTC	TNTC	TNTC	TNTC								
$10^{(-3)}$	TNTC	TNTC	TNTC	TNTC	161	180	177	173								
$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	13	27	26	22	TNTC	TNTC	TNTC	TNTC	210	210	190	203
$10^{(-5)}$	TNTC	TNTC	TNTC	TNTC	4	1	2	2	TNTC	TNTC	TNTC	TNTC	21	19	33	24
$10^{(-6)}$	86	75	89	83					47	30	35	37	3	3	4	3
$10^{(-7)}$									6	8	11					

Table A-6 (Continued)

Time (min)	100				100				120				120			
Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
$10^{(-2)}$					TNTC	TNTC	TNTC	TNTC					TNTC	TNTC	TNTC	TNTC
$10^{(-3)}$					94	190	117	134					TNTC	TNTC	TNTC	TNTC
$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	30	115	32	59					110	98	N/A	104
$10^{(-5)}$	170	145	200	172					TNTC	TNTC	TNTC	TNTC				
$10^{(-6)}$	25	32	26	28					61	49	50	53				
$10^{(-7)}$	2	6	1	3					8	2	4	5				

Table A-7 Results of ozone disinfection experiments of suspended HPC bacteria in 0.05 M, PH 6.9 phosphate buffer at 22°C (Gyürék and Finch, 1997)

Run No.	pH	Contact time	Ozone residual	Ct	Initial HPC	Final HPC	Inactivation
		min	mg/L	mg x min/L	in 1 mL	in 1 mL	Log-units
1	6.9	9.75	0.47	4.58	3,740,000	6,360	2.8
2	6.9	0.58	1.90	1.1	6,860,000	36,800	2.3
3	6.9	14.78	0.68	10.05	5,280,000	705	3.9
4	6.9	14.93	0.19	2.84	5,350,000	6,130	2.9
5	6.9	9.87	0.21	2.07	2,290,000	6,350	2.6
6	6.9	4.67	0.46	2.15	2,550,000	6,450	2.6
7	6.9	9.80	0.82	8.04	4,330,000	4,060	3
8	6.9	9.53	1.69	16.11	3,960,000	479	3.9
9	6.9	1.00	0.46	0.46	58,300	638	2

Table A-8 Results summary of ozone experiments of attached HPC bacteria in 0.05 pH 6.0 phosphate buffer at 22°C in this study

Run No.	pH	Contact time	Ozone residual	Ct	Initial HPC	Final HPC	Inactivation
		min	mg/L	mg x min/L	CFU/cm^2 N_0	CFU/cm^2 N	log (N_0/N)
1	6	15	1.076	13.36	1,280,000	680,000	0.275
2	6	15	0.911	11.33	2,173,333	693,333	0.608
3	6	60	0.965	56.48	600,000	1,333,333	0.611
4	6	33	2.649	78.07	3,600,000	64,000	1.653
5	6	120	1.132	138.3	11,066,667	29,333	2.167
6	6	110	1.144	126.2	4,933,333	320,000	1.521
7	6	100	1.237	122.7	3,733,333	78,667	1.676
8	6	120	2.05	236.4	7,066,667	138,667	1.707

Table A-9 Original data of ozone following by monochloramine disinfection experiments of biofilm HPC bacteria in 0.05M ODF phosphate buffer at 22°C.

Replicate	1								2							
Treatment	Before disinfection N_0				After disinfection N				Before disinfection N_0				After disinfection N			
Time (min)	10 min + 24 hrs				10 min + 24 hrs				10 min + 24 hrs				10 min + 24 hrs			
Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
$10^{(-2)}$													TNTC	TNTC	TNTC	TNTC
$10^{(-3)}$					2	8	2	4	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	7	5	8	7	TNTC	TNTC	TNTC	TNTC	52	23	6	27
$10^{(-5)}$	TNTC	TNTC	TNTC	TNTC	0	0	0	0	220	200	250	225	1	2	1	1
$10^{(-6)}$	47	38	40	42	0	0	0	0	48	50	46	48				
$10^{(-7)}$	3	5	11	6												
Time (min)	10 min + 24 hrs				10 min + 24 hrs				10 min + 24 hrs				10 min + 24 hrs			
Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
$10^{(-1)}$													TNTC	TNTC	TNTC	TNTC
$10^{(-2)}$					6	3	6	5					72	64	49	62
$10^{(-3)}$	TNTC	TNTC	TNTC	TNTC	0	1	1	1	TNTC	TNTC	TNTC	TNTC	7	12	12	10
$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	0	1	0	0	TNTC	TNTC	TNTC	TNTC	1	3	1	2
$10^{(-5)}$	TNTC	TNTC	TNTC	TNTC	0	0	0	0	61	220	246	176	0	0	0	0
$10^{(-6)}$	29	30	30	30												

Table A-9 (Continued)

Time (min)	120 min + 24 hrs				120 min + 24 hrs				120 min + 24 hrs				120 min + 24 hrs			
Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
$10^{(-2)}$					24	26	28	26					57	61	64	61
$10^{(-3)}$	TNTC	TNTC	TNTC	TNTC	1	4	6		TNTC	TNTC	TNTC	TNTC	9	8	10	9
$10^{(-4)}$	188	288	TNTC	TNTC	1	1	0		TNTC	TNTC	TNTC	TNTC	1	1	0	1
$10^{(-5)}$	61	66	69	65	0	0	1		108	123	118	116	0	0	0	0
$10^{(-6)}$	4	10	9	8					23	20	13	19				
Time (min)	120 min + 24 hrs				120 min + 24 hrs				120 min + 24 hrs				120 min + 24 hrs			
Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
$10^{(-2)}$					TNTC	TNTC	TNTC	TNTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
$10^{(-3)}$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	118	173	*	146	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
$10^{(-5)}$	123	156	144	141	80	60	*	70	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
$10^{(-6)}$	18	10	98	42					N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table A-10 Raw data from monochloramine disinfection experiments of biofilm HPC bacteria in 0.05M ODF phosphate buffer at 22°C.

Replicate	1								2							
Treatment	Before disinfection N_0				After disinfection N				Before disinfection N_0				After disinfection N			
Time (min)	6 hrs								6 hrs							
Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
$10^{(-2)}$													166	146	179	164
$10^{(-3)}$					76	50	70	65	TNTC	TNTC	TNTC	TNTC	26	20	18	21
$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	7	1	0	3	TNTC	TNTC	TNTC	TNTC	0	3	7	3
$10^{(-5)}$	220	180	210	203	1	1	0	1	TNTC	TNTC	TNTC	TNTC	0	1	0	0
$10^{(-6)}$	38	37	21	32	0	0	0	0	67	57	55	60				
$10^{(-7)}$	7	2	4	4												
Treatment	Before disinfection N_0				After disinfection N				Before disinfection N_0				After disinfection N			
Time (min)	24 hrs								24 hrs							
Dilutions	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
$10^{(0)}$																
$10^{(-1)}$																
$10^{(-2)}$					TNTC	TNTC	TNTC	TNTC					TNTC	TNTC	TNTC	TNTC
$10^{(-3)}$	TNTC	TNTC	TNTC	TNTC	46	50	46	47	TNTC	TNTC	TNTC	TNTC	21	23	9	18
$10^{(-4)}$	TNTC	TNTC	TNTC	TNTC	4	9	2	5	TNTC	TNTC	TNTC	TNTC	0	4	1	2
$10^{(-5)}$	99	126	108	111	1	0	1	1	116	95	80	97	0	0	0	0
$10^{(-6)}$	35	34	31	33					14	15	11	13				

Table A-11 Measurement of ozone residual

Date	Feb. 06	Disinfectant		Ozone				No. Of Coupon			3	Total CT (mg.min/l)			122.7
Time (min)	0	5	10	15	20	30	40	50	60	70	80	90	100	110	120
Temp. (° C)	20.3	20.4	20.5	20.7	20.8	20.9	21.1	21.2	21.2	21.2	21.2	21.3	21.3		
Abs. @ 600 1	0.463	0.358	0.335	0.314	0.326	0.326	0.333	0.319	0.326	0.360	0.327	0.300	0.374		
Abs. @ 600 2	0.463	0.358	0.335	0.314	0.326	0.326	0.333	0.319	0.326	0.36	0.327	0.300	0.374		
Abs.@ 600 Avg.	0.463	0.358	0.335	0.314	0.326	0.326	0.333	0.319	0.326	0.36	0.327	0.300	0.374		
Blank – Avg.		0.105	0.128	0.149	0.137	0.137	0.13	0.144	0.137	0.103	0.136	0.163	0.089		
Conc. Of Ozone Residual (mg/l)	0	1	1.219	1.419	1.305	1.305	1.238	1.371	1.305	0.981	1.295	1.552	0.848		
Ct (mg x min / l)		2.5	5.548	6.595	6.81	13.05	12.71	13.05	13.38	11.43	11.38	14.24	12		
Absorbance of Indigo Reagent II at 600nm = 0.166															
Concentration of O3 stock solution at 260nm = 2.222*14.5 = 32.2mg/L															

Date	Feb.16	Disinfectant		Ozone				No. Of Coupon			6	Total CT (mg.min/l)			236.4
Time (min)	0	5	10	15	20	30	40	50	60	70	80	90	100	110	120
Temp. (° C)	17.8	17.2	17.4	17.5	17.6	18.5	18.2	18.3	18.1	18.1	18.2	18.3	18.2	18.2	18.1
Abs. @ 600 1	0.472	0.175	0.194	0.229	0.260	0.386	0.225	0.198	0.250	0.271	0.208	0.252	0.302	0.312	0.332
Abs. @ 600 2	0.472	0.175	0.194	0.229	0.260	0.386	0.225	0.198	0.25	0.271	0.208	0.253	0.302	0.312	0.332
Abs.@ 600 Avg.	0.472	0.175	0.194	0.229	0.260	0.386	0.225	0.198	0.25	0.271	0.208	0.252	0.301	0.312	0.332
Blank – Avg.		0.297	0.278	0.243	0.212	0.086	0.247	0.274	0.222	0.201	0.264	0.22	0.17	0.16	0.14
Conc. Of Ozone Residual (mg/l)	0	2.829	2.648	2.314	2.019	0.819	2.352	2.610	2.114	1.914	2.514	2.095	1.619	1.524	1.333
Ct (mg x min / l)		7.07	13.69	12.40	10.83	14.19	15.86	24.81	23.62	20.14	22.14	23.05	18.57	15.71	14.29
Absorbance of Indigo Reagent II at 600nm = 0.180															
Concentration of O3 stock solution at 260nm = 1.626*14.5 = 24.07mg/L															

Table A-11 (Continued)

Date	mar.17	Disinfectant		Ozone				No. of Coupon			11	Total CT (mg.min/l)			83.9
Time (min)	0	5	10	15	20	30	33								
Temp. (° C)	19.3	19.2	18.5	18.7	18.9	19.2	19.8								
Abs. @ 600 1	0.464	0.165	0.121	0.138	0.171	0.232	0.288								
Abs. @ 600 2		0.165	0.121	0.138	0.171	0.232	0.288								
Abs.@ 600 Avg.		0.165	0.121	0.138	0.171	0.232	0.288								
Blank - Avg.		0.299	0.343	0.326	0.293	0.232	0.176								
Conc. of Ozone Residual (mg/l)	0	2.848	3.267	3.105	2.79	2.21	1.676								
CT (mg min / l)		7.12	15.29	15.93	14.74	25.00	5.83								
Absorbance of Indigo Reagent II at 600nm = 0.198															
Concentration of O3 stock solution at 260nm = 1.571*14.5 = 22.8 mg/L															

Date	May.02	Disinfectant		Ozone				No. of Coupon			1	Total CT (mg.min/l)			126.2
Time (min)	0	5	10	15	20	30	40	50	60	70	80	90	100	110	120
Temp. (° C)	19.3	19.3	19.6	19.6	20	20	20.4	20.5	20.7	21	21.2	21.2	21.3	21.2	
Abs. @ 600 1	0.476	0.358	0.390	0.373	0.347	0.383	0.260	0.332	0.386	0.35	0.322	0.366	0.384	0.377	
Abs. @ 600 2	0.476	0.358	0.388	0.373	0.347	0.383	0.26	0.333	0.386	0.35	0.322	0.366	0.384	0.377	
Abs.@ 600 Avg.	0.476	0.358	0.389	0.373	0.347	0.383	0.26	0.332	0.386	0.35	0.322	0.366	0.384	0.377	
Blank-A		0.118	0.087	0.103	0.129	0.093	0.216	0.144	0.09	0.126	0.154	0.11	0.092	0.099	
Conc. of Ozone Residual (mg/l)	0	1.124	0.829	0.981	1.229	0.886	2.057	1.371	0.857	1.2	1.467	1.048	0.876	0.943	
CT (mg min / l)		2.81	4.88	4.52	5.52	10.57	14.71	17.14	11.14	10.29	13.33	12.57	9.619	9.095	
Absorbance of Indigo Reagent II at 600nm = 0.192															
Concentration of O3 stock solution at 260nm = 2.458*14.5 = 35.64 mg/L															

Table A-11 (Continued)

Date	May.09	Disinfectant		Ozone				No. of Coupon			5	Total CT (mg.min/l)			56.48
Time (min)	0	5	10	15	20	30	40	50	60	70	80	90	100	110	120
Temp. (°C)	20.2	20.1	20.4	20.6	20.8	20.8	20.9	20.9	21.1						
Abs. @ 600 1	0.465	0.342	0.392	0.377	0.373	0.356	0.363	0.348	0.359						
Abs. @ 600 2	0.465	0.342	0.393	0.378	0.373	0.356	0.361	0.348	0.359						
Abs. @ 600 Avg.	0.465	0.342	0.392	0.377	0.373	0.356	0.362	0.348	0.359						
Blank-A		0.123	0.073	0.088	0.092	0.109	0.103	0.117	0.106						
Conc. of Ozone Residual (mg/l)	0	1.171	0.695	0.838	0.876	1.038	0.981	1.114	1.01						
CT (mg min / l)		2.93	4.67	3.83	4.29	9.57	10.10	10.48	10.62						
Absorbance of Indigo Reagent II at 600nm = 0.189															
Concentration of O3 stock solution at 260nm = 1.090*14.5 = 15.8 mg/L (adjust the generator)															

Date	May.16	Disinfectant		Ozone				No. of Coupon			12	Total CT (mg.min/l)			13.36
Time (min)	0	5	10	15	20	30	40	50	60	70	80	90	100	110	120
Temp. (°C)	20.0	20.3	20.4	20.5											
Abs. @ 600 1	0.459	0.360	0.336	0.342											
Abs. @ 600 2	0.459	0.360	0.337	0.342											
Abs. @ 600 Avg.	0.459	0.360	0.336	0.342											
Blank-A		0.099	0.123	0.117											
Conc. of Ozone Residual (mg/l)	0	0.943	1.171	1.114											
CT (mg min / l)		2.36	5.29	5.71											
Absorbance of Indigo Reagent II at 600nm = 0.183															
Concentration of O3 stock solution at 260nm = 1.331*14.5 = 19.3mg/L															

Table A-11 (Continued)

Date	May.24	Disinfectant		Ozone				No. of Coupon			4	Total CT (mg.min/l)			138.3
Time (min)	0	5	10	15	20	30	40	50	60	70	80	90	100	110	120
Temp. (° C)	20.2	20.4	20.6	20.5	20.7	20.8	20.8	20.8	21.0	21.2	21.2	21.1	21.0	20.8	21
Abs. @ 600 1	0.444	0.373	0.382	0.348	0.333	0.322	0.337	0.295	0.340	0.369	0.358	0.295	0.235	0.26	0.305
Abs. @ 600 2	0.444	0.373	0.382	0.347	0.333	0.322	0.337	0.297	0.340	0.369	0.358	0.295	0.235	0.26	0.305
Abs.@ 600 Avg.	0.444	0.373	0.382	0.347	0.333	0.322	0.337	0.296	0.340	0.369	0.358	0.295	0.235	0.26	0.305
Blank-A		0.071	0.062	0.097	0.111	0.122	0.107	0.148	0.104	0.075	0.086	0.149	0.209	0.184	0.139
Conc. of Ozone Residual (mg/l)	0	0.676	0.59	0.924	1.057	1.162	1.019	1.41	0.99	0.714	0.819	1.419	1.99	1.752	1.324
CT (mg min / l)		1.69	3.17	3.79	4.95	11.10	10.90	12.14	12.00	8.52	7.67	11.19	17.05	18.71	15.38
Absorbance of Indigo Reagent II at 600nm = 0.178															
Concentration of O3 stock solution at 260nm = 1.432*14.5 = 20.76mg/L															

Date	May.30	Disinfectant		Ozone			
Time (min)	0	5	10	15	20	30	40
Temp. (° C)	19.3	19.7	19.9	20			
Abs. @ 600 1	0.448	0.367	0.34	0.35			
Abs. @ 600 2	0.448	0.367	0.34	0.35			
Abs. @ 600 Avg.	0.448	0.367	0.34	0.35			
Blank-A		0.081	0.108	0.098			
Conc. of Ozone Residual (mg/l)	0	0.771	1.029	0.933			
CT (mg min / l)		1.93	4.5	4.90	11.33		
Absorbance of Indigo Reagent II at 600nm = 0.175							
Concentration of O3 stock solution at 260nm = 1.256*14.5 = 18.2mg/L							

Table A-11 (Continued)

Date	Jun.14	Disinfectant		Ozone				No. of Coupon			8	CT (mg.min/l)			123.7
Time (min)	0	5	10	15	20	30	40	50	60	70	80	90	100	110	120
Temp. (°C)	20.9	21.1	21.2	21.4	21.4	21.5	21.5	21.6	21.6	21.7	21.8	21.8	21.9	21.8	21.9
Abs. @ 600 1	0.444	0.330	0.358	0.317	0.323	0.322	0.288	0.308	0.328	0.344	0.361	0.36	0.356	0.329	0.360
Abs. @ 600 2	0.444	0.330	0.358	0.317	0.322	0.322	0.288	0.309	0.328	0.343	0.361	0.359	0.356	0.329	0.360
Abs. 600 Avg.	0.444	0.330	0.358	0.317	0.322	0.322	0.288	0.308	0.328	0.343	0.361	0.359	0.356	0.329	0.360
Blank-A		0.114	0.086	0.127	0.122	0.122	0.156	0.136	0.116	0.101	0.083	0.085	0.088	0.115	0.084
Conc. of Ozone Residual(mg/l)	0	1.08571	0.819	1.21	1.162	1.162	1.486	1.295	1.105	0.962	0.79	0.81	0.838	1.095	0.8
CT (mg min / l)		2.71429	4.762	5.071	5.929	11.62	13.24	13.9	12	10.33	8.762	8	8.238	9.667	9.476
Absorbance of Indigo Reagent II at 600nm = 0.172															
Concentration of O3 stock solution at 260nm = 1.692*14.5 = 24.5mg O2 /L															

Table A-12 Measurement of monochloramine residual

Date	May. 02			Date	May. 09		
Time (hour)	0	6		Time (hour)	0	6	24
Temp. (° C)	19.3	22.3		Temp. (° C)	19.3	22.3	22.2
Abs. @515 1	0.828	0.758		Abs. @515 1	0.828	0.758	0.66
Abs. @ 515 2	0.828	0.756		Abs. @ 515 2	0.828	0.756	0.658
Abs. @ 515 Avg.	0.828	0.757		Abs. @ 515 Avg.	0.828	0.757	0.659
Conc. of NH_2CL Residual (mg/l)	2.72	2.48		Conc. of NH_2CL Residual (mg/l)	2.72	2.48	2.16

Date	May. 16			Date	May. 24		
Time (hour)	0	6	24	Time (hour)	0	6	24
Temp. (° C)	17.6	22	22.3	Temp. (° C)	18.3	21.9	22
Abs. @515 1	0.843	0.774	0.717	Abs. @515 1	0.78	0.726	0.658
Abs. @ 515 2	0.844	0.774	0.717	Abs. @ 515 2	0.78	0.726	0.657
Abs. @ 515 Avg.	0.844	0.774	0.717	Abs. @ 515 Avg.	0.78	0.726	0.657
Conc. of NH_2CL Residual (mg/l)	2.77	2.54	2.35	Conc. of NH_2CL Residual (mg/l)	2.56	2.38	2.16

Date	May. 30			Date	Jun.14		
Time (hour)	0	6		Time (hour)	0	6	24
Temp. (° C)	18.5	21.3		Temp. (° C)	18.7	22.1	22.4
Abs. @515 1	0.726	0.694		Abs. @515 1	0.779	0.73	0.65
Abs. @ 515 2	0.726	0.693		Abs. @ 515 2	0.78	0.73	0.647
Abs. @ 515 Avg.	0.726	0.693		Abs. @ 515 Avg.	0.779	0.73	0.648
Conc. of NH_2CL Residual (mg/l)	2.38	2.27		Conc. of NH_2CL Residual (mg/l)	2.56	2.39	2.13